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(54) Title: METHODS FOR ASSAYING GENE IMPRINTING AND METHYLATED CpG ISLANDS

(57) Abstract: Genomic imprinting is a parent of origin-dependent gene silencing that involves marking of alleles in the germline and differential expression in somatic cells of the offspring. Imprinted genes and abnormal imprinting have been implicated in development, human disease, and embryonic stem cell transplantation. We have established a model system for genomic imprinting using pluripotent 8.5 d.p.c. mouse embryonic germ (EG) cell lines derived from an interspecific cross. We find that allele-specific imprinted gene expression has been lost in these cells. However, partial restoration of allele-specific silencing can occur for some imprinted genes after *in vitro* differentiation of EG cells into somatic cell lineages, indicating the presence of a gametic memory that is separable from allele-specific gene silencing. We have also generated a library containing most methylated CpG islands. A subset of these clones was analyzed and revealed a subdivision of methylated CpG islands into 4 distinct subtypes: CpG islands belonging to high copy number repeat families; unique CpG islands methylated in all tissues; unique methylated CpG islands that are unmethylated in the paternal germline; and unique CpG islands methylated in tumors. This approach identifies a *methylome* of methylated CpG islands throughout the genome.

WO 01/90313 A2

METHODS FOR ASSAYING GENE IMPRINTING AND METHYLATED CpG ISLANDS

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BACKGROUND OF THE INVENTION

Genomic imprinting is a parental origin-specific gene silencing that leads to differential expression of the two alleles of a gene in mammalian cells. Imprinting has attracted intense interest for several reasons: (i) Imprinting is by definition reversible and may be regulated over a large genomic domain (1). (ii) Imprinted genes and the imprinting mechanism itself are important in human birth defects and cancer (2). (iii) It has been suggested that imprinting cannot be reprogrammed without passage through the germline and thus constitutes a barrier to human embryonic stem cell transplantation (3).

Experimental studies of the timing and mechanism of genomic imprinting have been hampered by the fact that imprinting requires passage through the germline, analysis of which poses a difficult experimental target. Thus, there is a need in the art for an experimental model system which allows direct examination of allele-specific gene silencing in the dynamic process of genomic imprinting.

DNA methylation is central to many mammalian processes including embryonal development, X-inactivation, genomic imprinting, regulation of gene expression, and host defense against parasites, as well as abnormal processes such as carcinogenesis, fragile site

expression, and cytosine to thymine transition mutations. DNA methylation in mammals is achieved by the transfer of a methyl group from S-adenosyl-methionine to the C5 position of cytosine. This reaction is catalyzed by DNA methyltransferases and is specific to cytosines in CpG dinucleotides. 70% of all cytosines in CpG dinucleotides in the human genome are methylated and prone to deamination, resulting in a cytosine to thymine transition. This process leads to an overall reduction in the frequency of guanine and cytosine to about 40% of all nucleotides and a further reduction in the frequency of CpG dinucleotides to about a quarter of their expected frequency (35). The exception to this rule are CpG islands, that were first identified as HpaII tiny fragments (36), later to be defined as sequences of 1-2 kb with a GC content of above 50% and a frequency of CpG dinucleotides greater than 0.6 of their expected frequency (37). CpG islands have been estimated to constitute 1-2% of the mammalian genome (38), and are found around the promoters of all housekeeping genes, as well as in a less conserved position in 40% of tissue specific genes (39). The persistence of CpG dinucleotides in CpG islands is largely attributed to a general lack of methylation, regardless of expression status (reviewed in ref. 40).

The two exceptions to the rule of CpG islands being unmethylated in normal cells, are on the inactive X chromosome (41) and in association with imprinted genes (42,43). Genomic imprinting is the differential expression of the two parental alleles of a gene, and most imprinted genes are associated with at least one CpG island methylated uniquely on a specific parental chromosome (42). In addition, aberrant methylation of CpG islands has been observed in tumors and cultured cells, and it is thought to be a mechanism to silence tumor suppressor genes (44,45).

Numerous approaches have been used to identify CpG islands that are differentially methylated in specific cell types, such as tumor-normal pairs for cancer-related methylation

changes (46-48), or differential parental origin for imprinted genes (49-50). However, there was only one report of a systematic effort to identify CpG islands throughout the genome that might be normally methylated (51) using a methyl-CPG binding column. However, the resulting sequences were mainly dispersed repeats, ribosomal DNA and other repeated sequences with no characterization of unique, methylated CpG island.

There is a need in the art for identification of unique, methylated CpG islands so that imprinted genes can be identified.

SUMMARY OF THE INVENTION

One embodiment of the invention provides a method of forming embryonic germ cells useful as a model system for studying imprinting. A male and a female mammal of the same species are mated to form a pregnant female mammal. The male and the female mammals are sufficiently genetically divergent such that at least 50% of genes in resulting offspring have at least one sequence difference between alleles of said genes. An embryo is obtained from the pregnant female mammal at a stage of embryonic development between when 2-3 somites become visualizable and when gonads are recognizable. The embryo is dissected and cells of the embryo are dissociated. The dissociated cells are cultured to provide embryonic germ cell lines.

According to another embodiment of the invention a method is provided for inducing imprinting *in vitro*. Mammalian embryonic germ cells are cultured in suspension culture under conditions in which the embryonic germ cells differentiate. Expression of one or more imprintable genes changes from approximately equal biallelic to preferentially uniparental.

One aspect of the invention provides a method of inducing imprinting *in vivo*. One or more mammalian embryonic germ cells are injected into a nude mouse. The embryonic

germ cells differentiate and form a teratocarcinoma. Expression of one or more imprintable genes changes from approximately equal biallelic to preferentially uniparental.

Another aspect of the invention is a method of inducing imprinting *in vivo*. A mammalian embryonic germ cell is injected into a blastocyst of a mammal. The blastocyst is injected into a pseudopregnant mammal so that the blastocyst develops into a chimeric mammal. Expression of one or more imprintable genes in somatic cells derived from the embryonic germ cell becomes preferentially uniparental.

According to still another aspect of the invention an isolated and purified mammalian embryonic germ cell line is provided. It expresses one or more imprintable genes in a biparental fashion. It forms cells which express one or more imprintable genes in a biparental manner. It differentiates to form cells which express said one or more imprintable genes in a preferentially uniparental fashion.

According to another embodiment of the invention a method of testing substances as candidate drugs is provided. An isolated and purified mammalian embryonic germ cell line as described above is contacted with a test substance. Imprinting of one or more imprintable genes is assayed.

Another embodiment of the invention provides a method of testing substances as candidates drugs. Isolated and purified mammalian embryonic germ cell line as described above are contacted with a test substance. Methylation of one or more imprintable genes is assayed.

According to still another aspect of the invention a method is provided for making a chimeric animal which can be used as a model system for imprinting. A mammalian embryonic germ cell is transfected with a vector which expresses a detectable marker protein. The embryonic germ cell expresses one or more imprintable genes in a biparental manner.

The transfected mammalian embryonic germ cells is injected into a blastocyst of a mammal. The blastocyst is implanted into a pseudopregnant mammal. The blastocyst develops into a chimeric mammal. The chimeric mammal expresses the one or more imprintable genes in a preferentially uniparental fashion. The present invention also provides chimeric mammals made by the process.

Still another aspect of the invention provides a method for isolating methylated CpG islands. Eukaryotic genomic DNA is digested with a first restriction endonuclease which recognizes a recognition sequence found in A/T rich regions of DNA or found in CpG island-poor regions of DNA. The eukaryotic genomic DNA is digested with a second restriction endonuclease which recognizes a 4 base-pair sequence in unmethylated C/G rich regions. Fragments of at least 1 kb formed by the step of digesting are isolated and the fragments are inserted into bacterial vectors. Non-methylating, non-restricting bacteria are transformed with the bacterial vectors to propagate the vectors and render the fragments' progeny unmethylated. The unmethylated fragments are digested with a third restriction endonuclease which recognizes a sequence of at least 6 base pair in G/C rich regions. The resulting fragments are isolated and inserted into bacterial vectors to form a library of sequences which are enriched for sequences derived from methylated CpG islands in the eukaryotic genome.

Also provided by the present invention are a library of fragments which are enriched at least 100-fold in methylated CpG islands relative to total genomic DNA.

Further aspects of the invention provide a method for testing substances as candidate drugs. A nude mouse which has been injected with an embryonic germs cell to form a teratoma is contacted with a test substance. A test substance is identified as a candidate drug if it inhibits the growth of the teratoma or causes regression of the teratoma.

The present invention also provides a method of providing an assessment of risk of developing cancer. Methylation status is determined in a sample of a patient for a CpG island selected from the group identified in Table 2 (below). The methylation status of the CpG island is compared to that found in a control group of healthy individuals. The patient is identified as having an increased risk of developing cancer if methylation status of the CpG island is perturbed relative to the methylation status in the control group.

Another aspect of the invention is a method of providing diagnostic information relative to cancer. Methylation status of a CpG island selected from the group identified in Table 2 is determined in a sample of a tissue of a patient suspected of being neoplastic. The methylation status of the CpG island is compared to that found in a control sample of said tissue which is apparently normal. The patient is identified as having an increased risk of developing cancer if methylation status of the CpG island is perturbed relative to the methylation status in the control sample.

According to yet another aspect of the invention an isolated and purified methylated CpG island is provided which is selected from those shown in Table 2.

Still another aspect of the invention provides a method of identifying imprinted genes. A gene is identified which is within about 2 million base pairs of a CpG island identified in Table 2 in the human genome. One determines whether the gene is preferentially uniparentally expressed. The gene is identified as an imprinted gene if it is preferentially uniparentally expressed.

According to another aspect of the invention an isolated and purified methylated CpG island is provided. Surprisingly, the island is methylated in both maternal and paternal alleles of a human.

Another aspect of the invention provides an isolated and purified methylated CpG island which is biallelically methylated in some humans and not biallelically methylated in other humans. The methylated CpG island thus comprises a methylation polymorphism.

The present invention thus provides the art with tools and methods for accessing imprinted genes and using them for detecting birth defects, diabetes, and cancers associated with aberrant imprinting.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1. Experimental design. E8.5 F1 (129/SvEv x CAST/Ei) embryos were dissected near the base of the allantois to initiate PGC cultures from which EG cell lines were established. EG cell lines were differentiated *in vitro* by either of several methods, injected subcutaneously into athymic nude mice to form teratocarcinoma, or transfected with a GFP vector and injected into the blastocysts of C57BL/6 to generate chimeric mice, from which differentiated cells were purified by FACS.

Figure 2A -2F. Characterization of mouse interspecific EG cell lines. (Fig. 2A) Colony of EG cell line SJEG-1 cultured on a feeder layer of STO cells, viewed by phase contrast microscopy. (Fig. 2B) EG colonies stained positive for alkaline phosphatase. (Fig. 2C) Embryoid bodies formed upon spontaneous differentiation on plastic, viewed by phase contrast microscopy. (Fig. 2D) A rhythmically contracting muscle bundle formed by differentiation of SJEG-1 cells transfected with α mMHCneo vector. (Fig. 2E) Erythrocytes, epithelia, and (Fig. 2F) striated muscles in H&E sections of teratocarcinoma formed after injection of SJEG-1 cells into nude mice. Scale bars: 10 μ m in Fig. 2A, Fig. 2B, and Fig. 2D; 100 μ m in Fig. 2C, Fig. 2E, and Fig. 2F.

Figure 3A and 3B. Partial imprinting establishment of EG cells induced by spontaneous *in vitro* differentiation on plastic. RNA and DNA were prepared at varying times during differentiation. (Fig. 3A) SSCP analysis of allele-specific expression of Kvlqt1, Igf2, and L23mrp. Paternal (Castaneus) and maternal (129) bands are indicated. The upper band is a nonspecific PCR product. (Fig. 3B) Changes in ratio of parental allele expression of Kvlqt1, Igf2, H19, Snrpn, Igf2r, and L23mrp. Means and standard deviations are calculated from 4-7 experiments each.

Figure 4A and 4B. Independence of imprinting establishment from method of *in vitro* differentiation. (Fig. 4A) SNUPE analysis of allele-specific expression of Snrpn. SJEG-1 cells were differentiated with all-trans retinoic acid (RA), dimethyl sulfoxide (DMSO), and in methylcellulose medium. Cells were harvested at 12 and 20 days of differentiation. (Fig. 4B) SSCP analysis of allele-specific expression of Kvlqt1 in α MHCneo-transfected SJEG-1 cells that were differentiated into cardiac myocytes.

Figure 5A -5E. Nearly complete imprinting of EG cells after *in vivo* differentiation. (Fig. 5A) FACS analysis of SJEG-1 and SJEG-1/GFP18-1 cell lines for GFP fluorescence intensity. SJEG-1/GFP18-1 was derived from SJEG-1 by transfection with pEGFP-N3 vector and injected into the blastocyst of C57BL/6. (Fig. 5B) FACS analysis of spleen cells isolated from a chimeric mouse and a non-chimeric littermate. Cells with fluorescence intensity greater than 40 units were collected, since the fluorescence intensity of >99.9% of cells derived from donor embryos fell below 30 units. (Fig. 5C, Fig. 5D, Fig. 5E) Analysis of allele-specific expression of (Fig. 5C) Kvlqt1 and (Fig. 5D) Igf2 by SSCP, and (Fig. 5E)

Snrpn by SNUPE, in GFP+ spleen cells obtained from chimeric mice. Paternal (Castaneus) and maternal (129) bands are indicated. The upper constant band in (Fig. 5D) is a nonspecific PCR product.

Figure 6A and 6B. *De novo* establishment of allele-specific methylation of H19 and Igf2 in EG cells by *in vitro* differentiation. (Fig. 6A) Analysis of H19 DMR. Genomic DNA was digested with EcoR I (E), Msc I (M), and Hpa II (H), and hybridized with a 450 bp probe, resulting in a 2.6 kb band representing methylated DNA, and a 1.74 kb band representing unmethylated DNA. The ratios of unmethylated to methylated bands were 4.3, 2.3, 1.3, 1.2, and 0.83, at 0, 6, 10, 13, and 16 days, respectively. (Fig. 6B) Analysis of Igf2 DMR2. Genomic DNA was digested with BamH I (B) and Hpa II (h), and hybridized with a 640 bp probe resulting in a 2.45 kb band representing methylated DNA, and several lower molecular weight bands representing unmethylated DNA. An unrelated cross-hybridizing band (C) variably appears as described previously (16). The ratios of methylated to unmethylated bands were 4, 4.8, 1.6, and 0.9, at 0, 10, 13, and 16 days, respectively.

Figure 7A-7D. Nearly complete imprinting in differentiated human EG cells. (Fig. 7A) Monolayer culture of differentiated human EG cells (LV.EB) obtained from previously reported human EG cultures (21) under phase contrast microscopy. Scale bar, 10 μ m. (Fig. 7B) Nearly complete monoallelic expression of IGF2 in differentiated human EG cells. PCR products of genomic DNA were digested with Apa I revealing heterozygosity for A (236 bp) and B (173 bp) alleles. Digestion of RT-PCR products (+RT) shows nearly complete preferential expression of the A allele, with no product in the absence of reverse transcriptase (-RT). (Fig. 7C) Complete monoallelic expression of H19 gene in differentiated human EG

cells. Digestion of PCR products with Alu I resulted in both digested (128/100 bp doublet) and undigested (228 bp) alleles in genomic DNA, and only the undigested allele (148 bp) in cDNA. (Fig. 7D) Analysis of H19 DMR of differentiated human EG cells. Genomic DNA of differentiated EG cells (LV.EB) and a control tissue was digested with Sma I (H) and Pst I (P) and hybridized to a 1 kb probe, resulting in a 1.6 kb band representing methylated DNA, and a 1.0 kb band representing unmethylated DNA.

Figure 8. Model of genomic imprinting in EG cells. For some imprinted genes, EG cells derived from e8.5 embryos retain a gametic memory of the parental origin of the chromosome (colored boxes), although allele-specific silencing and methylation (black dots) are lost. On differentiation into somatic cells, the EG cells re-establish allele-specific silencing and methylation. For EG cells derived from older embryos, this gametic memory has been erased, so that there is no change in biallelic expression (green arrows) or DNA methylation on differentiation into somatic cells.

Figure 9. Overall strategy for cloning methylated CpG islands. Male genomic DNA from a Wilms tumor was digested with Hpa II and Mse I, fragments ≥ 1 kb in size were subcloned into a modified pGEM-4Z vector and transformed into XL2-Blue MRF', resulting in an expected 10 X enrichment for methylated CpG islands, that was confirmed by Southern hybridization. Library DNA was then digested with Eag I, and fragments between 100 bp and 1500 bp were subcloned into pBC and transformed into XL1-Blue MRF' resulting in an expected 800 X enrichment for methylated CpG islands. Black ellipse depicts a methylated CpG island, clear ellipse depicts an unmethylated CpG island. In step 1, thick arrowheads above the line depict Mse I sites (TTAA) and below the line depict unmethylated Hpa II sites (CCGG). In step 2, thick arrowheads depict Eag I sites (CGGCCG). Enrichment estimates were based on an *in silico* analysis of frequencies of Mse I, Hpa II, and other CpG-rich restriction endonucleases including Eag I, in CpG islands vs. non CpG island DNA: Mse I

fragments ≥ 1 kb in size included 77 % of CpG islands and 8% of non-CpG island DNA ($0.77/0.08 = 10$ X enrichment). In the second step, 43% of the set of CpG islands would have been cloned by Eag I and thus for a two-step cloning using Mse I and Eag I, the fraction of methylated CpG islands expected is $0.43 \times 0.77 = 0.33$. The expected 800 X enrichment is derived from the expected fraction of CpG islands after an Eag I digest (0.028) divided by the initial estimated fraction of methylated CpG islands based on the only known normally methylated autosomal CpG islands, i.e. those associated with imprinted genes.

Figure 10. Methylation of SVA retroposons. DNA was digested with Mse I (M), Mse I + Hpa II (MH), or Mse I+Msp I (MM), electrophoresed on a 1.5% agarose gel, transferred to a nylon membrane and hybridized to a probe unique to the SVA element, SVA-U. LI: liver; LU: lung; fKI: fetal kidney; fLIM: fetal limb; SP: sperm; PT: parthenogenetic tumor (dysgerminoma).

Figure 11A -11C. Methylation of MCI-S in normal tissues. DNA from various tissues was digested with Mse I (M), Mse I+Hpa II (MH), or Mse I+Msp I (MM), electrophoresed on a 1.5% agarose gel, transferred to a nylon membrane and hybridized with MCI-S clones. Fig. 11A) MCI-S are methylated in blood. Fig. 11B) MCI-S/1-19 is methylated in fetal and adult somatic tissues. Fig. 11C) MCI-S are methylated in uniparental and germline tissues. fCNS: fetal central nervous system; fKI: fetal kidney; fLU: fetal lung; fSK: fetal skin; BR: brain; CO: colon; KI: kidney; LI: liver; OT: ovarian teratoma; CHM: complete hydatidiform mole.

Figure 12A -12C. Methylation of MCI-D in normal tissues. Tissue DNA was treated as described in Figure 3 and hybridized with MCI-D clones. Fig. 12A) MCI-D are methylated in blood. Fig. 12B) MCI-D/2-78 is methylated in fetal and adult somatic tissues. Fig. 12C) MCI-D methylation in uniparental and germline tissues: MCI-D are methylated in maternally derived tissues and germline, unmethylated in sperm and complete hydatidiform mole, and half-methylated in adult testis. fCNS:

fetal central nervous system; fGU: fetal gut; fHE: fetal heart; fKI: fetal kidney; fLU: fetal lung; BR: brain; CO: colon; HE: heart; KI: kidney; LI: liver; OT: ovarian teratoma; CHM: complete hydatidiform mole; OV: ovary; fOV: fetal ovary; TE: testis; fTE: fetal testis.

Figure 13. Variable methylation of MCI-T/2-d10 in normal tissue and Wilms tumor. DNA from normal blood, the tumor that was used to construct the Mse I library (denoted WT*), and two pairs of matched Wilms tumor and normal kidney from the same patients, was treated as described in Figure 11 and hybridized with MCI-T/2-d10.

Figure 14. Sequence of isolated CpG islands are shown which are not available in public databases.

DETAILED DESCRIPTION OF THE DRAWINGS

We have derived highly polymorphic pluripotent EG cell lines from an interspecific mouse cross, and have shown that these cells lack allele-specific expression and methylation, but acquire these features after *in vitro* and *in vivo* differentiation into somatic cell lineages. These results have three important implications. First, these EG cell lines represent the first *in vitro* model system in which genomic imprinting can be followed dynamically and the two alleles can be distinguished. This system significantly enhances the identification and characterization of trans and cis-acting elements that modify imprinting, and it also confers the advantages of extending such investigations into an *in vivo* setting.

Second, these results demonstrate that gametic allele memory and allele-specific methylation are separable mechanisms. Our data suggest a model in which undifferentiated EG cells obtained from e8.5 embryos retain a memory of their own parental origin even in the absence of allele-specific silencing and methylation (Fig. 8). On differentiation into

somatic cell lineages, this gametic memory becomes manifest (Fig. 8), as imprinted genes acquired allele-specific expression and methylation. In EG cells derived from later stage embryos, this gametic memory is lost (the PGCs from which the EG cells are derived would eventually become reprogrammed according to their own gender), and thus late stage EG cells or PGCs are unable to undergo allele-specific silencing and methylation on differentiation (18). Even in our early stage EG cells, this gametic memory was not preserved for all imprinted genes, as *Igf2r* was unable to attain imprinting after differentiation. This idea is also consistent with the observation that pre-implantation embryos may not show monoallelic expression of all imprinted genes (24).

This model also has important implications for understanding loss of imprinting (LOI) in cancer (2). We have found that the normal pattern of allele-specific methylation can be restored to at least some tumor cells with loss-of-imprinting (LOI), suggesting that some gametic memory is retained in these cells (25). Similarly, Mitsuya et al. have found that human chromosomes introduced into mouse hybrids by microcell-mediated transfer can lose allele-specific expression but reacquire it after the cells are treated with differentiating agents (26). These observations are consistent with our proposal that a gametic memory is distinct from allele-specific expression and methylation at known DMRs, as we propose here. While the molecular basis of this gametic memory is unknown, candidate mechanisms could include histone acetylation, special chromatin structures, or DNA methylation elsewhere along the chromosome.

Third, since early EG cells did not for the most part lose a gametic imprinting mark, despite biallelic expression in those cells prior to differentiation, we hypothesized that differentiated cell lineages derived from early human EG cells would also show comparatively normal imprinting. This hypothesis was contrary to predictions (19) based on

studies of late mouse EG cells or PGCs (18). Our examination of differentiated human EG-derived cells demonstrated normal imprinting at the level of both gene expression and DNA methylation. Thus, genomic imprinting is unlikely to be a barrier to human embryonic stem cell transplantation.

We have also identified methylated CpG islands present in normal tissues (termed MCI). There have been systematic efforts to identify unique CpG islands differentially methylated in tumors (46-48) but no such successful efforts have been described for normally methylated CpG islands. While such sequences may have been suspected, this study represents their first systematic identification in normal tissues, and as such represents a first step toward defining a "methylome", i.e. the distribution of methylation patterns layered on the distribution of genes in the genome.

MCI sequences appear to fall within distinct biological subgroups. We divided the MCI sequences into four categories, based on their copy number and methylation pattern. The first group, MCI-R, is clearly the most abundant, and comprises high copy number sequences such as the SVA element, and the intergenic and internal spacer sequences of ribosomal genes. Methylation of one of these sequences, the rDNA nontranscribed spacer, was previously found after genomic purification from a methyl-CpG binding protein column (51), and one wonders whether the large number of these sequences obscured the identification of unique MCI's. The methylation of high copy number MCI sequences is not surprising, as it is consistent with the hypothesis of that CpG methylation arose as a host defense mechanism (63). This is particularly true of the SVA element, which is a high copy number retroposon.

Of greater interest in this study are the unique CpG islands methylated in normal tissues. There has been great interest in CpG island sequences because of their presumed

function in regulation of expression of housekeeping genes (40), their potential involvement in silencing genes in tumors (44,45), and their role in providing a parental origin-specific mark to imprinted genes (42). Our prediction that 1-2% of CpG islands are methylated in normal tissues will likely alter our perspective on CpG islands in general. An important direction of future effort will be to add to the number of known methylated CpG islands. There are several alternative approaches for generating additional second libraries from the Mse I library, although the simplest approach for identifying additional MCIs may be high throughput sequencing of the Mse I library itself. We estimate that the Mse I library contains approximately 77% of the MCI sequences, and we believe that all of the CpG islands within the Mse I library represent such sequences.

We were surprised by the large number of unique methylated CpG islands we were able to identify using a restriction endonuclease-based cloning strategy that eliminated most of the MCI-R sequences from the library. The two largest classes of these unique methylated CpG islands, MCI-S and MCI-D, appear to have different properties, suggesting that they may serve distinct potential functional roles. Specifically, the MCI-S sequences were localized to high isochore regions near the ends of chromosomes, and the MCI-D sequences generally showed a more centromeric localization within low isochore regions. It is remarkable that the MCI-S, which are ubiquitously methylated, even in sperm, retain their high CpG content, which also suggests that they may serve an important role. That role, however, would not appear to be gene silencing, since most of the MCI-S were within the body of transcriptionally active genes.

The MCI-D sequences are particularly interesting for further study, because of their apparent differential methylation in the germline. In particular, these sequences may mark imprinted gene regions, as at least two of these sequences in the Eag I library were found

within imprinted genes, namely *IGF2R* and *HYMAI*. Furthermore, most imprinted genes appear to lie within low isochore regions (*PLAGL1*, *IGF2R*, *PEG1/MEST*, *SNRPN*, *PEG3*, *GNAS*, unpublished data), like the MCI-D sequences. An intriguing possibility is that a subset of low isochore domains, marked with MCI-D sequences, harbor such genes.

Also surprisingly, most of these unique sequences were not tumor-specific (MCI-T) but were also methylated in normal tissues. We suspect that the MCI-T may represent a comparatively small fraction of the total number of unique methylated CpG islands. One possibility that will be the subject of further study is that the MCI-T may include sequences that are variably methylated in the population, such as MCI-T/2-d10. This is an intriguing idea because it suggests that the methylome might contribute to polymorphic variation in the population, which is consistent with the idea that methylation mutations may be more common in outbred populations than in laboratory strains (64).

Imprinting as used herein is the preferential expression of a specific parental allele, maternal or paternal. Typically it is associated with the modification of a specific parental allele, such as by DNA methylation, histone acetylation, histone phosphorylation, or histone methylation. Imprinting can be assessed using any method known in the art for determining expression from a particular allele. Such techniques include without limitation pyrosequencing for high throughput assaying, MALDI-TOF mass spectrometry, allele specific oligonucleotide DNA microarray, Hot-stop PCR (Uejima et al., *Nat. Genet.* 2000, 4:375-6), SSCP (single stranded conformational polymorphism assay), QS (quantitative sequencing), SNUPE (Single nucleotide primer extension), and allele-specific ligation assay. Unimprinted genes are typically expressed in an approximately equal biallelic fashion, whereas imprinted genes display preferential expression of a specific parental allele. Approximately equal biallelic expression may be as disparate as about 40 % : 60 %,.

preferably from about 45 %: 55 %, more preferably from about 47.5 % : 52.5 %. Expression differences greater than this, such as 30 %:70 %, 20 %:80 %, 10 %:90 %, and 5 %: 95 % are considered preferential expression of a specific parental allele.

Methylated CpG islands which are repetitive (MCI-R) can be used as portable sites of genetic recombination, as indications of past chromosomal rearrangements or as indications of past insertion element-created mutations. Most CpG dinucleotides within a methylated CpG island contain a methylated 5-position on the pyrimidine ring of cytosine. The methylation level within a CpG island is believed to be quite high, with at least 75 %, 80 %, 90 %, 95 %, or even 98% of the cytosine residues being methylated. Functionally, the methylated CpG islands survive the isolation procedure which involves restriction with a restriction endonuclease which cleaves at unmethylated CpG dinucleotides. Methylated CpG islands which are differentially methylated among maternal-derived and paternal-derived tissues (MCI-D) can be used as markers of the locations of imprinted genes.

Typically, MCI-D are located within imprinted genes are adjacent to imprinted genes. Adjacency is within 2×10^6 base pairs, preferably within 1×10^6 base pairs, more preferably within 0.5×10^6 base pairs. MCI-S and MCI-T, methylated CpG islands which are expressed similarly in uniparental tissues and those which are differentially expressed in tumors and normal tissues, can be used as methylation polymorphism markers in the population. Thus they can be used as sequence polymorphisms, forensically, diagnostically, and predictively as risk factors for disease traits.

Embryonic germ cells are useful as a dynamic model system for studying imprinting. The ability to induce imprinting permits the analysis of factors which stimulate or inhibit the process. The factors can be endogenous or exogenously applied. It is desirable to use parental animals which are of the same species yet which are sufficiently genetically

divergent such that at least 50% of genes in resulting offspring have at least one sequence difference between alleles of said genes. More preferably at least 60 %, 70 %, 75 %, 80 %, 90 %, or 95 % of the maternal and paternal genes in the offspring will be detectably different. This greatly facilitates analysis of imprinting by rendering most genes amenable to analysis of differential allelic expression. Suitable mammals which can be used include without limitation mice, rats, hamsters, guinea pigs, rabbits, goats, cows, sheep, pigs, horses, dogs, and cats.

Embryos are desirably removed from the pregnant female mammal at a stage of embryonic development between when 2-3 somites become visualizable and when gonads are recognizable. In mice, this stage is between day 7 and 10 post conception. Obtaining embryos at such an early stage is believed to be beneficial in obtaining cells which have many genes which are not yet imprinted. Embryos are dissected and cultured, preferably on feeder cell layers. The posterior third of the embryo can be dissected and used to form dissociated cells. Alternatively, the genital ridge of the embryo is dissected out and used to form dissociated cells. Still another alternative method dissects out gonads of the embryo to form dissociated cells.

Once cell lines have been obtained they can be used for various assays and tests. The cell lines express one or more imprintable genes in an approximately equal biparental fashion, form cells which express one or more imprintable genes in an approximately equal biparental manner, and differentiate to form cells which express said one or more imprintable genes in a preferentially uniparental fashion. The assays for imprinting can be done *in vitro* or *in vivo* as is desired by the practitioner. In one assay, the mammalian embryonic germ cells are grown in suspension culture under conditions in which the embryonic germ cells differentiate. The differentiated cells may or may not form an embryoid body. Upon

differentiation expression of one or more imprintable genes changes from approximately equal biallelic to preferentially uniparental. Differentiation can be induced by growth on plastic in the absence of feeder cells, by growth in the presence of dimethylsulfoxide, by growth in the presence of retinoic acid, by growth on a methyl-cellulose containing medium, or any other method known in the art. According to one particularly preferred method the germ cells contain a selectable marker under transcriptional control of a tissue-specific promoter, and the germ cells are subjected to selection conditions to select for germ cells which have differentiated into a lineage which activates the tissue-specific promoter.

A number of techniques are available for inducing and observing imprinting *in vivo* using the cell lines of the present invention. The mammalian embryonic germ cells can be injected into a nude mouse in which it will form a teratocarcinoma. One or more imprintable genes change from approximately equal biallelic to preferentially uniparental expression upon formation of the teratocarcinoma. Another way to achieve imprinting in an *in vivo* model is to inject a mammalian embryonic germ cell into a blastocyst of a mammal. The blastocyst is then implanted into a pseudopregnant mammal so that the blastocyst develops into a chimeric mammal, *i.e.*, its somatic cells are not genetically identical. Expression of one or more imprintable genes in somatic cells derived from the embryonic germ cell becomes preferentially uniparental. The germ cells used for formation of teratocarcinomas or chimeric blastocysts can optionally be transfected with a vector which expresses a detectable marker protein. This makes distinguishing among the cells of the mammal a simpler exercise.

Imprinting can be assayed directly in any of the models of the invention by detecting parental allele specific expression. Alternatively, a surrogate for such expression can be used

such as cytosine methylation, histone acetylation, histone phosphorylation, histone methylation. Methods for detecting such modifications are known in the art.

Test substances used to contact with the cell lines or chimeric mammals of the present invention can be any natural, synthetic, or semisynthetic substance, whether a pure compound or a mixture of compounds. The test substances can be compounds or drugs which are known to have one or more biological effects, or substances which are not known to have any biological or physiological effects. If the test animal contains a teratoma, one can identify a test substance as a candidate drug if it inhibits the growth of the teratoma or causes regression of the teratoma. Techniques for assessing the growth of a teratoma or regression of a teratoma are well known in the art.

Methylated CpG islands can be isolated using a scheme as outlined in Figure 9. Any restriction endonucleases can be used which have the desired properties specified. The properties are based on the frequency of cleavage sites, and the preference of the cleavage sites for being in G/C or A/T rich regions. The CpG islands can be isolated from genomic DNA from males or females, from tumor or normal cells. Any type of tumor or normal tissue can be used as a source of cells. Once such methylated CpG islands are isolated, they can be used for a number of different techniques. In one, they are tested to identify sequences which are differentially methylated between maternal and paternal chromosomes. In another technique they are tested to identify sequences which are differentially methylated between hydatidiform moles and teratomas. In another technique they are mapped to a genomic region. The CpG islands can be used to identify an imprinted gene adjacent to the methylated CpG island, as methylated CpG islands are markers for such genes. If a CpG island is found to map to the same region as a disease which is preferentially transmitted by one parent, an imprinted gene in the region can be identified as a candidate gene involved in transmitting the

disease. The CpG islands can be used to screen populations of individuals for methylation. A sequence which is differentially methylated between individuals is a methylation polymorphism which can be used to identify individuals.

Practice of the disclosed method for isolating CpG islands creates libraries which are enriched at least 100-fold, at least 250-fold, at least 500-fold, or at least 750-fold in methylated CpG islands relative to total genomic DNA. Preferably each library of fragments will contain at least 25, at least 50, or at least 75 distinct members.

The particular CpG islands which have been found using the method of the present invention are disclosed in Table 2. These particular CpG islands can be used to assess risk of developing cancer. Perturbed methylation of CpG islands relative to sequences in a control group of healthy individuals suggests that the individual being tested are at increased risk of developing cancer. Any number of CpG islands can be tested in such a method, but preferably at least 2, 5, 10, or 15 such islands will be tested. An increased risk of developing cancer is determined if at least 1 of 2, 3 of 5, 6 of 10, or 8 of 15 of the CpG islands have perturbed methylation status relative to control group. Similarly aberrant methylation of CpG islands can be determined where the methylation in a suspect tissue sample of a patient is compared to the methylation in an ostensibly healthy tissue sample of the patient.

CpG islands can be used to identify genes which are within about 2 million base pairs of a CpG island identified in Table 2 in the human genome. The genes are preferably within 1 million base pairs, and more preferably within 500,000 base pairs. If the gene is preferentially uniparentally expressed, then it is identified as an imprinted gene.

EXAMPLES

Example 1

We used 129/SvEv mice as the mothers in the cross. We chose CAST/Ei (*Mus musculus castaneus*) mice, separated from 129/SvEv by 5 million years in evolution, as the father in the cross, providing an average of one polymorphic marker per 400 bp of transcribed sequence. The experimental strategy is summarized in Figure 1, and it allows differentiation *in vitro* by a variety of mechanisms, including targeted differentiation using a selectable construct, and differentiation *in vivo* using chimeric mice.

Forty EG cell lines were derived from primordial germ cells (PGCs) of 8.5 day embryos (4), as determined by colony morphology and positive alkaline phosphatase staining (Fig. 2A,B), and four of these lines were characterized in detail (termed SJEG-1, 2, 7, and 15). These EG cell lines formed embryoid bodies after *in vitro* differentiation (Fig. 2C,D), teratocarcinomas in nude mice (Fig. 2E, F), and generated chimeric mice when injected into the blastocyst of C57BL/6 mice (5). One male line was also used for subsequent germline transmission (5). Most of the imprinting studies were done on lines SJEG-1, 2, and 7.

Example 2

Partial establishment of imprinting *in vitro*. In order to distinguish the two alleles of imprinted genes in these EG cell lines, we identified transcribed polymorphisms distinguishing 129/SvEv and CAST/Ei in 5 imprinted genes, Kvlqt1, Snrpn, Igf2, H19, and Igf2r, as well as the nonimprinted gene L23mrp as a negative control. For each gene, an assay for allele-specific expression was then developed, as described in Table 1.

Table 1. Transcribed polymorphisms and assay methods for allele-specific gene expression of EG cells derived from mouse interspecific cross.

Gene	Polymorphism			Assay Method
	CAST/Ei ¹	129/SvEv	Position ²	
Kvlqt1	TCC <u>C</u> TGC	TCC <u>A</u> TGC	1823	SSCP ³
Igf2	GCA <u>A</u> TTC	GCAG <u>T</u> TTC	777	SSCP ³
H19	CTT <u>G</u> GAG	CTT <u>T</u> GAG	1593	QS ⁴
Snrpn	CTA <u>T</u> AAT	CTA <u>C</u> AAT	915	SNuPE ⁵
Igf2r	ATC <u>G</u> ATG	ATC <u>A</u> ATG	1549	SNuPE ⁵
L23mrp	ACC <u>C</u> GAG	ACCT <u>T</u> GAG	407	SSCP ³

¹ Polymorphisms were identified by direct sequencing of CAST/Ei genomic DNA. 129/SvEv sequence was identical to known *Mus musculus musculus* sequence in GenBank, except that Kvlqt1 sequence was unavailable and done here.

² From first nucleotide of cDNA

³ Single strand conformation polymorphism (27).

⁴ Quantitative sequencing (28).

⁵ Single nucleotide primer extension (29).

Kvlqt1 shows preferential expression of the maternal allele throughout development in this strain background (6). Prior to somatic differentiation of EG cells *in vitro*, Kvlqt1 showed approximately equal expression of the two alleles (Fig. 3A). After differentiation by replating on plastic in the absence of a feeder cell layer, Kvlqt1 showed clear preferential expression of the maternal allele, which increased to a 6:1 ratio by day 16 (Fig. 3A), and this result was seen in all three cell lines tested (Fig. 3B). Like Kvlqt1, Igf2 showed approximately equal biallelic expression of the two parental alleles prior to differentiation (Fig. 3A). However, after EG cell differentiation, unlike Kvlqt1, which showed preferential allele-specific expression in the same parental direction as F1 offspring, Igf2 showed allele-specific expression but in opposite direction to the F1 offspring. Thus, differentiated EG cells showed preferential expression of the maternal allele of Igf2 (Fig. 3A). While this was a surprising observation, it was consistent among different cell lines (Fig. 3B). The expression of the maternal allele of IGF2 is also consistent with an observation of allele reversal in embryonic stem (ES) cells (7). This may be a property of pluripotent embryonic stem cells (although note that in contrast to EG cells, imprinting shows little or no change in ES cells (7)).

H19 normally shows reciprocal allele-specific expression to IGF2, perhaps due to competition for a shared enhancer (8). Consistent with this pattern, H19 exhibited approximately equal expression of the two parental alleles before differentiation, and preferential expression of the paternal allele after differentiation, changing from a ratio of 1:1 to 3:1 after differentiation (Fig. 3B). Snrpn, which is preferentially expressed from the paternal allele in somatic cells (9), also showed equal biallelic expression in undifferentiated EG cells (Fig. 3B). After differentiation,

Snrpn showed preferential expression of the normally expressed paternal allele, at a ratio of 3:1 (Fig. 3B). In contrast, Igf2r showed approximately equal biallelic expression both before and after differentiation, suggesting that for this gene, the gametic mark had been completely erased in EG cells (Fig. 3B).

As a negative control, we analyzed the nonimprinted gene L23mrp, which is just outside of a contiguous imprinted gene domain that includes Igf2, H19, and Kvlqt1 (10). In contrast to Igf2, H19, and Kvlqt1, L23mrp showed equal biallelic expression of the two parental alleles both before and after *in vitro* differentiation (Fig. 3A,B). Furthermore, the ratio of allele-specific expression of the imprinted genes after differentiation differed significantly from that of L23mrp ($p < 0.01$, two-tailed t-test). In summary, *in vitro* differentiation partially restored imprinting to EG cells.

Example 3

Imprinting was independent of differentiation method. In order to determine whether allele-specific expression in EG cells was caused by differentiation *in vitro*, or by the specific treatment used to differentiate EG cells, we repeated these experiments by differentiating the cells in 3 other ways (4): differentiation in methylcellulose medium; treatment with retinoic acid; and treatment with dimethyl sulfoxide. In all cases, the results were identical to those seen on spontaneous differentiation on plastic in the absence of a feeder cell layer. For example, Snrpn showed equal biallelic expression of the two parental alleles prior to differentiation, and preferential expression of the paternal allele after differentiation in all cases, but with slight variation in the final ratio of parental alleles (Fig. 4A).

Embryoid bodies that result from *in vitro* differentiation of EG cells show considerable cellular heterogeneity, and not all of the cells are differentiated. In order

to determine whether allele-specific expression would arise during differentiation down a specific cell lineage pathway, we used a genetic selection strategy to obtain lineage-specific EG cell differentiation. We transfected EG cells with a vector containing the neo selectable marker gene under the control of a mouse α -cardiac myosin heavy chain gene promoter (11). Clones of transfected EG cells remained undifferentiated, and showed equal biallelic expression of Kvlqt1, Igf2, H19, Snrpn, Igf2r and L23mrp (Fig. 4B and data not shown). Differentiation of transfected EG cells under G418 selection produced a network of rhythmically contracting myocyte bundles in culture (11) (Fig. 2D). Examination of these cells for allele-specific expression showed preferential allele expression similar to that seen using other differentiation approaches, but with a slightly greater ratio of allele-specific expression. For example, Kvlqt1 achieved a 9:1 ratio of maternal to paternal allele expression after cardiac myocyte-specific differentiation *in vitro* (Fig. 4B). Thus, establishment of imprinting was due to differentiation itself, and not to the specific methods used to induce it.

Example 4

Nearly complete imprinting establishment after differentiation of EG cells *in vivo*. To verify that the changes in imprinting we observed *in vitro* also occurred during natural differentiation *in vivo*, we took advantage of the pluripotency of our EG cell lines to generate mouse chimeras. In order to purify cells derived from these EG cells after *in vivo* differentiation in chimeric mice, we first transfected EG cells with a vector containing a modified GFP gene under the control of the CMV promoter (5) (Fig. 5A). We then injected the cells into C57BL/6 blastocysts, which were introduced into pseudopregnant mice and allowed to develop to term (5). Spleens were removed from chimeras, and the EG-derived GFP(+) cells were purified by

fluorescence-activated cell sorting (FACS) to 99% homogeneity (Fig. 5B). Purity of EG-derived cells isolated from the chimeric mice was confirmed by measuring the allele ratio in genomic DNA for polymorphisms that distinguish the two strains (data not shown).

Analysis of imprinting of EG-derived cells isolated after *in vivo* differentiation in chimeric mice indicated that all of the imprinted genes studied showed the same pattern of allele-specific expression found after *in vitro* differentiation. However, after *in vivo* differentiation, the degree of allele-specific expression was nearly complete. Thus, Kvlqt1 showed equal biallelic expression after transfection of the pEGFP-N3 vector and prior to blastocyst injection, and monoallelic expression of the maternal allele after *in vivo* differentiation in three separate chimeric mice (Fig. 5C). Similarly, Igf2 showed monoallelic expression of the maternal allele in two separate chimeric mice and nearly monoallelic expression ($>10:1$) in a third (Fig. 5D). H19 also showed monoallelic expression of the paternal allele, the same allele preferentially expressed after *in vitro* differentiation (data not shown). Finally, Snrpn exhibited predominant expression of the paternal allele (4:1 ratio) after *in vivo* differentiation. As a control, L23mrp showed equal biallelic expression after *in vivo* differentiation (data not shown). Thus, *in vivo* differentiation of EG cells caused nearly complete establishment of imprint-specific expression.

Example 5

Establishment of differential DNA methylation during *in vitro* differentiation of EG cells. From all of the above experiments, it is clear that these EG cell chromosomes retain some memory of their parental origin, but they do not manifest this memory as allele-specific expression until the cells are differentiated. DNA methylation has been shown previously to play a role in genomic imprinting,

because mice deficient in DNA methyltransferase I show loss of imprinting (12). In order to determine whether DNA methylation represents the mechanism of the gametic mark, we analyzed the methylation status of two previously well-characterized differentially methylated regions (DMR).

Differential methylation in the H19 gene DMR, located -4 to -2 kb upstream of the transcriptional start site, is established in the gamete and stably maintained during early development (13). Our analysis of undifferentiated EG cells revealed a hypomethylated pattern, at a ratio of 4.3:1 unmethylated to methylated bands (Fig. 6A). This result was consistent with the biallelic pattern of H19 expression in undifferentiated EG cells (Fig. 3B), since methylation of the H19 DMR is associated with allele-specific silencing (14). However, with *in vitro* differentiation, H19 acquired a typical half-methylated pattern, similar to that seen in the parental and F1 mice, with a 1:1 ratio of unmethylated to methylated bands (Fig. 6A). This change in methylation reflected well the change in expression from approximately biallelic to predominantly monoallelic in these cells after differentiation. To further determine which parental allele of H19 became methylated after *in vitro* differentiation, we analyzed the allele composition of methylated H19 DMR using a previously described method (13). Our analysis of differentiated EG cells revealed that the half-methylation pattern described above (Fig. 6A) was due to methylation of the non-expressed allele (data not shown). Thus, the methylation was allele-specific and related to silencing of the H19 gene during differentiation.

Igf2 DMR2, within exon 6, is known to be the more closely linked DMR to Igf2 imprinting (15). We analyzed its methylation in EG cells by methods previously described (16). Analysis of undifferentiated EG cells revealed a hypermethylated pattern, at a ratio of 4:1 methylated to unmethylated bands (Fig. 6B), consistent with

the biallelic expression of Igf2 in undifferentiated cells (Fig. 3A,B), since the methylation of Igf2 DMR2 is normally associated with the expressed allele (15). With *in vitro* differentiation, Igf2 acquired a half-methylated pattern, with a 1:1 ratio of methylated to unmethylated bands (Fig. 6B), consistent with the predominantly monoallelic expression of Igf2 after differentiation (Fig. 3A,B). Thus, DNA methylation reflected the pattern of gene expression of both Igf2 and H19, with a nonimprinted pattern of DNA methylation before differentiation, and an imprinted pattern after differentiation.

Example 6

Nearly complete imprinting in differentiated human EG cells. Pluripotent human EG cell cultures have recently been derived (17). The potential therapeutic use of these cells in medicine has received considerable attention, since they can be employed as an unlimited source for a variety of tissues used in human transplantation therapy. However, some recent experiments using late mouse EG cells (e12.5) and PGCs (e14.5-16.5) suggested that genomic imprinting could not be established, and lack of imprinting is associated with developmental abnormalities and embryonic mortality (18). These results have raised widespread public concern over the feasibility of human EG cells for therapeutic use (19).

Because of these concerns, we endeavored to determine whether human EG cells can achieve genomic imprinting after differentiation, like mouse EG cells. We examined genomic imprinting in a differentiated monolayer culture of lineage-restricted cell types (20) (Fig. 7A), derived from a human EG culture reported previously (17). IGF2 was examined using an Apa I polymorphism in exon 9 (21). While Apa I digestion revealed two alleles in genomic DNA, analysis of cDNA showed a nearly complete monoallelic expression pattern (Fig. 7B), indicating a

nearly complete establishment of imprinting of IGF2 gene after *in vitro* differentiation of a human EG culture. H19 was then examined using an Alu I polymorphism in exon 5 (22). While Alu I digestion revealed two alleles in genomic DNA, analysis of cDNA showed a complete monoallelic expression pattern (Fig. 7C), indicating complete establishment of imprinting of H19 after *in vitro* differentiation of human EG culture.

We further examined the methylation pattern of the H19 DMR (23) in differentiated human EG cells. A double digestion of genomic DNA using Pst I and the methylation-sensitive enzyme Sma I revealed a 1.6 kb methylated and a 1.0 kb unmethylated allele in control human tissue samples (Fig. 7D). Analysis of differentiated EG-derived cells showed the same methylation pattern seen in normal human tissues (Fig. 7D), indicating the establishment of a normal imprinting pattern in human EG-derived cells.

Example 7

Experimental Design. We chose a restriction enzyme-based strategy for isolating methylated CpG islands over a PCR-based strategy, to avoid known problems of amplification bias against GC-rich sequences, and in order to obtain larger clone inserts than would be possible by a PCR-based approach. The source of DNA was a Wilms tumor from a male, to avoid cloning methylated CpG islands from the inactive X chromosome, and because this approach would identify either normally methylated CpG islands or those methylated specifically in tumors. The specific enzymes were chosen by an *in silico* analysis of genomic sequences containing CpG islands. This analysis suggested a two-step approach (described in detail in Fig. 9). The first step involves digestion with Mse I and Hpa II, followed by gel purification

of fragments ≥ 1 kb in length. This step was predicted to enrich approximately 10-fold for CpG islands (enrichment was confirmed by a Southern blot, data not shown), while eliminating all unmethylated CpG islands because of the methylcytosine sensitivity of Hpa II. This "Mse I library" was cloned into the restriction-negative strain XL2-Blue MRF' to avoid bacterial digestion of methylated genomic DNA. CpG islands were further selected by digesting Mse I library DNA with Eag I and subcloning, providing a total expected 800-fold enrichment for CpG islands in this "Eag I" library (see Fig. 9 brief description for details). Taking together the estimated library size and unique clones in it, with the predicted enrichment from the specific enzymatic strategy that was used, we estimated the total number of unique methylated CpG islands throughout the genome to be approximately 800, representing 1-2% of the total number of CpG islands.

Construction of the Mse I library. DNA from a male Wilms' tumor sample was isolated as described (52). 200 μ g of DNA were digested overnight with 1000 units of Hpa II (LTI) followed by a five hour digest with 600 units of Mse I (NEB), according to the manufacturer's conditions, and the volume was reduced using a SpeedVac concentrator (Savant). In order to select for fragments ≥ 1 kb, the digest was passed through a size selection CHROMA-SPIN+TE-400 column (Clontech). Fragments between 1-9 kb were purified from a 0.8% gel by electroelution and passed through an Elutip-D column (S&S). The eluate was ethanol precipitated, cloned into the compatible Nde I site of pGEM-4Z, which was first modified to abolish the Sma I site, transformed into the competent cells of the restriction-deficient strain XL2-Blue MRF' (Stratagene), and plated onto LB-Ampicillin agar plates. Library DNA was prepared directly from plates using a plasmid Maxi kit (Qiagen).

Construction of the Eag I libraries. 100 µg of the Mse I library DNA were digested with 1,000 u of Eag I (NEB) according to the manufacturer's conditions. The digest was ethanol precipitated, and 100 to 1500 bp fragments were size-selected by purification from a 1.5% agarose gel, cloned into the Eag I site of pBC (Stratagene), and transformed into XL1-Blue MRF' (Stratagene). DNA from individual colonies was prepared using a Perfect Prep kit (Eppendorf). In order to eliminate MCI-R sequences (Methylated CpG Island Repetitive, see results) from the final Eag I library, 3.5 µg of the Mse I library was purified, and half was digested with Acc I and half with Tth III1, pooled and digested with Dra III, Sal I, and Asc I, then re-transformed into XL2-Blue MRF'. This step eliminated >90% of the MCI-R sequences, while retaining approximately 30% of the MCI-S and MCI-D sequences (MCI-same in uniparental tissues, MCI-different in uniparental tissues, respectively, see results). Eag I libraries were prepared as described above, after gel purification from three overlapping fractions, 100-700 bp, 400-1000 bp, 700-1500 bp, termed ES-1, 2, and 3, respectively.

DNA Sequencing. DNA sequencing was performed using an ABI 377 automated sequencer following protocols recommended by the manufacturer (Perkin-Elmer). The sequences were analyzed by a BLAST search (53) of the NR, dbEST, dbGSS, dbHTGS, and dbSTS databases, and by GRAIL analysis. Chromosomal localization was performed by electronic PCR (ePCR, NCBI), or in some cases without matches using the GeneBridge 4 radiation hybrids panel (Research Genetics).

Southern hybridization. Genomic DNA was digested with Mse I alone or Mse I together with a methylcytosine-sensitive (Hpa II, LTI, or Sma I, NEB) or

methyl-insensitive (Msp I or Xma I, NEB) restriction endonuclease according to the manufacturer's conditions. Southern hybridization was performed as described (54).

Example 8

A class of high copy number methylated CpG islands. Our primary goal was to identify unique methylated CpG islands throughout the genome. However, it quickly became apparent that most of the clones in the Eag I library represented high copy number methylated CpG islands. The majority of these were derived from a sequence termed SVA, which constituted 70% of the Eag I library, and that was not previously known to be methylated. The little-known SVA retroposon contains a GC-rich VNTR region, which embodies a CpG island, between an Alu-derived region and an LTR-derived region, only three such elements had previously been described (55-57), although their methylation has not been characterized. We designed a probe, termed SVA-U, unique to the SVA and present in all of the SVA elements, to analyze copy number and methylation of this sequence in genomic DNA. The copy number was estimated to be 5000 per haploid genome (data not shown, L.S.-A. and A.P.F., in preparation). The SVA elements were found to be completely methylated in all adult somatic tissues examined, including peripheral blood lymphocytes, kidney, adrenal, liver and lung, as well as fetal tissues including kidney, limb, and lung (Fig. 10). However, in germinal tissues SVA elements were hypomethylated but not completely unmethylated. This methylation pattern was consistent with a retroposon methylation pattern, where a group of active elements is unmethylated in the germ line and maintains a high GC content, whereas in somatic tissues the element is methylated and silenced. A somewhat less abundant high copy repeat, representing an additional 20% of the Eag I library corresponded to the nontranscribed intergenic spacer of

ribosomal DNA, which was a known methylated repetitive sequence (58). A third high copy methylated sequence was the ribosomal DNA internal transcribed spacer and the 28S gene, comprising an estimated 5% of the Eag I library, suggesting that ribosomal gene methylation may be more extensive than was previously suspected. In summary approximately 25% of the Eag I library was accounted for by ribosomal DNA sequences, and 95% of the Eag I library by ribosomal DNA and SVA together. For convenience, we term this class of methylated CpG islands MCI-R (Methylated CpG Island-Repetitive).

Example 9

Identification of Unique Methylated CpG Islands. One of the advantages of our restriction enzyme-based two-step approach is that we could use it to eliminate the high copy number sequences described above. Toward this end, we again performed an *in silico* analysis to identify combinations of restriction endonucleases that could be used on the Mse I library, to selectively eliminate the two common high copy number methylated CpG islands, and an Eag I library was re-constructed following this procedure. This approach allowed us to uncover unique methylated CpG islands that might otherwise have been obscured.

After eliminating redundant clones, sixty-two unique clones were characterized in detail. All of the sequences were GC-rich, i.e. with a measured $(C + G) / N > 50\%$, and they ranged in GC content from 55 to 79%. Forty-five (73%) of the clones showed an observed to expected CpG ratio > 0.6 , meeting the formal definitional requirement of a CpG island. Thirty of these CpG islands were then characterized by detailed genomic analysis, including radiation hybrid mapping of clones not within the known database, and analysis of methylation in somatic and

germline tissues and in ovarian teratomas (OT) and complete hydatidiform moles (CHM), which are of uniparental maternal and paternal origin, respectively.

While the sequences recovered in this manner were predicted to be methylated, we confirmed this assumption by direct examination of genomic DNA. Furthermore, as the original source of material was a Wilms tumor DNA sample, we had no a priori knowledge about the methylation of these sequences in normal tissue. Surprisingly, most were methylated normally. More specifically, this analysis revealed that all of the sequences represented methylated CpG islands, and they could be divided into 3 major groups. The largest group consisted of sequences methylated in all tissues examined, including fetal and adult somatic tissues, ovarian teratomas (OT), complete hydatidiform moles (CHM), and sperm. For example, clone 1-41 showed in blood an identical pattern after Mse I + Hpa II digestion, as after Mse I digestion alone, compared to Mse I + Msp I digestion which cut regardless of methylation (Fig. 11A). This was true for other somatic tissues, as well as for ovarian teratoma, hydatidiform mole, and sperm (Fig. 11B,C). Altogether, half of the unique methylated CpG islands fell within this category, which we term **MCI-S** (Methylated CpG Island-Similar in uniparental tissues).

The second largest group, approximately 30% of the unique clones, were methylated in normal somatic tissues, and unmethylated in complete hydatidiform mole (CHM), which are uniparentally derived from the male germline, as well as in sperm. For example, clone 2-78 showed an identical pattern after Mse I + Hpa II digestion, as after Mse I digestion alone, in blood and other somatic tissues (Fig. 12A,B). However, clone 2-78 showed complete digestion after Hpa II treatment of sperm and hydatidiform mole DNA, similar to the pattern seen after Msp I digestion (Fig. 12C). We termed this category **MCI-D** (Methylated CpG Island-Different in

uniparental tissues). All of the MCI-D sequences were methylated in OT and not CHM.

The final group, approximately 10 % of the unique clones, were unmethylated in normal tissue but methylated in tumors. For example, clone 2-d10 showed an identical methylation pattern in blood DNA after Mse I + Hpa II digestion as was seen after Mse I + Msp I digestion. However, Wilms tumor DNA, from which the Mse I library had been constructed, was fully methylated (Fig. 13). Consistent with our nomenclature, this category is termed MCI-T (Methylated CpG Island—Tumors). Though the MCI-T sequences were identified by virtue of their being methylated in tumor tissue, they may represent sequences of polymorphic methylation in the population, as a second individual showed methylation of 2-d10 in both tumor and normal tissues and a third showed methylation in neither tumor nor normal tissues (Fig. 13).

Example 10

Chromosomal and isochore localization of unique methylated CpG islands. The remainder of the studies described here were performed on the two classes of unique CpG islands that are methylated in normal tissues, namely MCI-S and MCI-D. We first asked whether these sequences were found in a unique location in the genome or were distributed more generally. Surprisingly, there was a striking difference in localization within the genome of the MCI-S and MCI-D sequences. Virtually all of the MCI-S sequences were localized near the ends of chromosomes, either on the last or the penultimate subband of the chromosome on which it resided (Table 2). In contrast, 70% of MCI-D sequences were localized more centromerically. This difference was highly statistically significant ($p < 0.01$, Fisher's

exact test). The association of MCI-S sequences near the ends of chromosomes is consistent with an observation of densely methylated GC-rich sequences near telomeres, although that study did not describe methylated CpG islands (51).

Table 2. Characteristics of MCI-S and MCI-D Sequences.

Name	Accession	Gene	Expression	Chromosome	Isochore
MCI-S/1-5	AL161774	-	NA	13qtel	H2 (54%)
MCI-S/1-19	AF084481	WFS1	Br,Bra,Co,Ey,He,Ki,Li,Lu,Ly,Ov,Pa,Pl,Te,Ut	4p15	H2 (52%)
MCI-S/1-30	AC008267	-	NA	7q11-21	H1 (46%)
MCI-S/1-41	NM_018104	FLJ10474	Br,Lu,Mu,Pr,Ut	ND	
MCI-S 2-e3	AC010958	-	NA	ND	H1 (49%)
MCI-S 2-h1	U60110	N-SGA-b	Br,Bra,Lu,Pa,Pl,Pr,St,Te,Ut	17q25	H1 (51%)
MCI-S/3-110	AC023786	-	NA	ND	H1 (51%)
MCI-S/3-12	AL157939	-	NA	10q26	H2 (56%)
MCI-S/3-20	AA001705	EST	Retina	ND	
MCI-S/3-c10	AK025954	FLJ22301	Br,Bra,Co,Ey,Ge,He,Ki,Lu,Ly,Mu,Ov,Pa,Pl,Te	1q44	
MCI-S/4-f3	Hs.155647	EST	Br,Co,Ma,Pr,Te	19p13	H3 (66%)

MCI-S/4-g6	AI361872	EST	CGAP-CLL	ND
MCI-D/1-13	AP001403	-	NA	18q23 L (43%)
MCI-D/1-20	AL161645	-	NA	10q26 H1 (48%)
MCI-D/1-21	NM_016651	LOC51339	infant/fetal brain	14q21 L (41%)
MCI-D/2-4	U43342	NFAT	activated T cells	20q13 H1 (48%)
MCI-D/2-42	AC026454	-	NA	16p11 H1 (49%)
MCI-D/2-48	Hs.202088	EST	CGAP-Lung	9p11-12 L (42%)
MCI-D/2-78	AW090822	EST	Testis, CGAP-Brain	18q12 L (41%)
MCI-D/2-e4	AC012191	-	NA	8q21 L (39%)
MCI-D 3-30	Hs.148365	5' of EST	fetal Lung/Testis/GCB	11q24 H1 (45%)
MCI-D 3-d4	AF241534	HYMAI	fetal Heart, CGAP-CLL, Ge	6q24 L (39%)

Expression data was derived from experimental data (not shown) as well as from information in UniGene. Chromosome localization was derived from ePCR and radiation hybrids mapping. Isochore determination was according to the composition of the genomic sequence harboring the clone; Accession - GenBank accession; NA-not applicable, ND-not done, Br: brain, Bra: breast, Co: colon, Ey: eye, Ge: germ cell, He: heart, Ki:

kidney, Li: liver, Lu: lung, Ly: lymph, Mu: muscle, Ov: ovary, Pa: parathyroid, Pl: placenta, Pr: prostate, St: stomach, Te: testis, To: tonsil, Ut: uterus CGAP: Cancer Gene Anatomy Project, CLL: Chronic Lymphocytic Leukemia, GCB: Germinal Center B-Cells.

We also questioned whether, in addition to their apparent chromosomal segregation, the MCI-D and MCI-S sequences localized within compartments of differing genomic composition, i.e. isochores, which are regions of several hundred kb of relatively homogeneous GC composition (59). This analysis showed a striking segregation of MCI-D and MCI-S sequences. Approximately 75% of the MCI-S sequences fell within high isochore regions ($G+C \geq 50\%$), as might be expected from the high GC content of methylated CpG islands. Surprisingly, however, all of the MCI-D sequences fell within low isochore regions ($G+C < 50\%$), i.e. of relatively low GC content, despite the high GC content of the MCI-D sequences themselves (Table 1). This difference, like the chromosomal localization was also highly statistically significant ($p < 0.01$, Fisher's exact test). Taken together, the comparison of MCI-S and MCI-D localization suggest that they may lie within distinct chromosomal and/or isochore compartments.

Example 11

Relationship of unique methylated CpG islands to genes. Most of the MCI-D and MCI-S sequences were localized within or near the coding sequence of known genes or of anonymous ESTs within the GenBank database. These genes serve a wide variety of functions, including the *wolframin* gene, a transmembrane protein involved in congenital diabetes; *sulphamidase*, a lysosomal enzyme involved in Sanfilippo syndrome (MPS-IIIa); a cDNA similar to the gene for the extracellular matrix protein tenascin; and an EST adjacent to the Peutz-Jeghers syndrome gene *STK11* (Table 2). Half of the MCI-S and one of the MCI-D sequences corresponded to unique or very low copy number variable number tandem repeat (VNTR)

sequences. The location of the CpG islands within these genes appeared to differ between the MCI-S and MCI-D sequences, although this difference was not statistically significant. Three of six MCI-D sequences were localized within the promoter or contained the predicted transcriptional start site. For example, MCI-D/2-78 matched EST AW090822, including the start of a 546 amino acid long ORF and a promoter predicted by GENSCAN just upstream of this sequence, and MCI-D/3-d4 was within the promoter and first exon of the *HYMAI* gene. In contrast, none of 7 MCI-S sequences were found to include the start site of transcription. For example, MCI-S/1-19 was within the last exon of the *wolframin* gene, and MCI-S/2-h1 was within the 5-6 exons of the *sulphamidase* gene. Finally, some of the MCI-D sequences may lie within or near imprinted genes, consistent with their differential methylation in uniparental tissues. For example, the *IGF2R* gene, which contains an Eag I site, was identified in the Eag I library (data not shown), consistent with the observation that one allele is methylated in normal cells. In addition, MSI-D/3-d4, which like other MSI-D sequences was methylated differentially in ovarian teratomas and hydatidiform moles, differed from most other MSI-D sequences in that it was only partially methylated in somatic tissues. Interestingly, this sequence was found to lie within the promoter and first exon of the *HYMAI* gene, which has recently also been demonstrated to be imprinted (60). Thus, a subset of MCI-D sequences may mark the location of imprinted genes.

Example 12

Protocol for EG Cell Line Derivation

Media

1. STO medium

DMEM supplemented with 10% FBS and Pen-Strep. Used for STO, SI⁴-m220, SI⁴-X9D3 culture.

2. EG medium

DMEM with high glucose (4.5 g/liter) supplemented with 15% FBS (performance tested), non-essential amino acid (0.01 mM), L-glutamine (2 mM), Pen-Strep, and 2-mercaptoethanol (0.1 mM).

Feeder layer preparation

1. Gelatin-coated 24-well plate preparation.

Add 0.1% gelatin in dH₂O into each wells and incubate for about one hour.

Wash the well twice with PBS. Allow the well filled with PBS or dH₂O.

2. Prepare feeder layer.

1) STO culture

STO cells are used as feeder layers for EG derivation and long term culture. Normally STO culture is maintained in 10 cm dish in STO media. Culture must be split before reaching 85% confluence. Irradiation resistance of the maintained culture needs to be tested after a certain period of time. Should cells surviving irradiation found, throw away the culture and thaw a new vial of cells.

2) Prepare feeder layer

a. Trypsinize STO from culture the day before dissecting embryo. Suspend cells in culture media in 50 cc tubes. Irradiate cells for 4000 rads. Count the cells and pellet.

Resuspend cells in media at 1.5×10^5 cells/ml. Add 1 ml (1.5×10^5 cells) of cell suspension into each well of gelatin-coated 24-well plate. Allow cells settle on the bottom overnight.

b. 2 hours before embryo dissection, change media in the wells into EG media supplemented with LIF (1000 U/ml), bFGF (1 ng/ml), and murine SCF (stem cell factor) (60 ng/ml).

Mice mating

Natural mating is setup for 129/SvEv female and mus. Castaneous male. Male must be older than 7 weeks and female must be between 8-18 weeks.

Put 2-3 females into a male cage in which only one male mouse is kept at the end of the day. Check plug on females next morning. Separate plugged females into new cages (one in each) and label the cage indicating the male partner.

Embryo Dissection

Dissect out the posterior third of the embryo from 8.5 dpc embryo.

Dissect out the genital ridge from 10.5 dpc embryo.

Dissect out the pair of gonads from 12.5 dpc embryo.

Primary culture

1. Pool all dissected tissue fragments into a 15 cc tube. Rinse with PBS once. Dissociate cells by adding 1 ml of 0.25% tyrosine/1mM EDTA solution and gently pipetting up and down for 2.5 min. Then add 5 ml of EG media and keep pipetting up and down for about 2 min. Pellet cells at 1000 rpm for 10 min. Resuspend cells into an appropriate volume (for 8.5 dpc, 200 ul/embryo; 10.5 and 12.5 dpc, 1 ml/embryo) of EG media supplemented with LIF (1000 U/ml), bFGF (1 ng/ml), and murine SCF (stem cell factor) (60 ng/ml). Add 100 ul into each feeder layer coated wells of 24-well plate.

2. Plate dissociated cell suspension into at least two separate plates. One with only a few wells plated for monitoring the survival and proliferation of PGCs in culture. Others with most or all of wells plated for EG derivation.
3. After 6 days, some of the wells are stained for alkaline phosphatase each day in order to assess the survival and growth of PGCs.

Secondary culture and line cloning

1. At 9th days, prepare feeder layer plates.
2. After 10 days, cultures are trypsinized and replated: 2 hours before trypsinization, change media for feeder layer plate into EG medium. Wash wells with PBS twice, and add 100 ul of 0.25% trypsin/1mM EDTA into each well. Incubate plates at 37°C for 2 min. Add 1 ml of EG media into each well and pipette up and down in the well. Collect trypsinized cultures of all wells into a 15 cc tube, pellet cells and resuspend cells into appropriate volume (1 ml/well) of EG media supplemented with LIF (1000 U/ml). Add 1 ml into each well of prepared feeder layer plate.
3. Monitor the appearance of colonies in culture every day.
4. When most colonies expand into unaided visible sizes, trypsinize the culture with 0.05% trypsin/EDTA and isolate floating colonies from the media. Isolated colonies are subjected to microdrop trepsinization (0.25% trypsin/EDTA) and plated into feeder layer of 24-well plates in EG media supplemented with LIF (1000 U/ml).
5. After two rounds of colony cloning, lines can be passed in 5 cm culture dish without further cloning.

Example 13

EG Cell Staining Protocol

Stage-specific mouse embryonic antigen-1 staining

1. Culture EG cells on STO feeder layer on a chamber slide (Nunc).
2. Wash culture twice with PBS containing 2% calf serum and 0.1% sodium azide.
3. Incubate culture with mouse monoclonal antibody (TG-1) against stage-specific mouse embryonic antigen-1 (at least 1:30 dilution) on ice for 30 min. (Ab from Dr. Peter Donovan in NCI)
4. After washed with PBS, culture are incubated for 30 min with FITC-conjugated Fab' fragment of goat anti-mouse IgG (H+L) (Cappel, 1:5 dilution) on ice.
5. Wash culture with PBS. Fix culture in 4% paraformaldehyde before staining for AP.

Alkaline phosphatase activity staining

Use leukocyte alkaline phosphatase kit (catalog No. 85L-3R) from SIGMA and follow the accompanying protocol.

Example 14

Differentiation Essay for EG cells

In vitro differentiation

Protocol I (Natural differentiation)

1. EG culture on feeder layer is trypsinized (0.05% trypsin EDTA) lightly and pipetted gently to generate small clumps of cells. Separate the EG cells from the irradiated STO cells as written below.
2. Transfer cell clumps into bacteriological plastic dishes and allow cell clumps to grow in suspension for 5 to 7 days. Most of clumps differentiate into simple embryoid bodies, with a single outer layer of extraembryonic ectoderm cells.
3. Return embryoid bodies back to tissue culture plastic dishes. Embryoid bodies will attach and give rise to a variety of cell types over two weeks.

Separate EG cells from STO feeder layer cells

For all the following protocols, EG cultures are trypsinized (0.25% trypsin/EDTA) and single cell suspension is created. Plate cells into 10 cm tissue culture dish at 37°C for 1.5 hr to allow feeder layer cells attach the bottom. Replate the media into another plate for an additional 1.5 hr. Then collect media and pellet cells.

Protocol II (DMSO induced differentiation as aggregates)

1. Resuspend cells into RA differentiation medium (DMEM supplemented with 1% dimethyl sulfoxide (DMSO), 10% FBS, L-Glutamine, Peniciline-Streptomycin) and transfer into bacteriological dishes.
2. After 4 days, transfer cell aggregates into tissue culture dishes and culture with regular medium.

Protocol III (RA induced differentiation as aggregates)

1. Resuspend cells into RA differentiation medium (DMEM supplemented with 0.3 μ M all-trans retinoic acid, 10% FBS, L-Glutamine, Peniciline-Streptomycin) and transfer into bacteriological dishes.
2. After 4 days, transfer cell aggregates into tissue culture dishes and culture with regular medium.

Protocol IV (Differentiation in methylcellulose medium)

1. Count EG cells and resuspend EG cells in methylcellulose medium* at a concentration of 3.5×10^5 cells/ml. Transfer 10 ml into each 10 cm bacteriological dish.

2. At day 4, split each dish into 2 dishes and grow for another 10 days with medium replaced daily.

* Methylcellulose medium (500 ml): Weight 3.7 g of NaHCO_3 and mix with 10 g of BRL DMEM salt (pack for 1 liter media). Dissolve salts into 86 ml water and pH to 6.9. Mix 20 ml of concentrated salt solution with 268 ml of DMEM, 50 ml FBS, 5 ml each of non-essential a.a., 2.3 ml of L-glutamine, 5 ml of pen-strep. at 100X concentrations, and 4.1 ul of 100% 2-mercaptoethanol. Filter the solution through 0.2 micrometre filter. Add 150 ml of 2.2% (w/v) aqueous methylcellulose (Sigma, viscosity of 2% aqueous solution equal to 400 centipoises), mix and store at 4° for 1 hr before use.

Preparation of 2.2% aqueous methylcellulose: Add 11 g of methylcellulose powder into bottle and add water to 500 ml. Stir the solution in cold room overnight. Put bottle in microwave and boil the solution three times (be careful not to spill the content). Tighten the cap right after the last boiling and leave the bottle in cold room overnight. Store in refrigerator.

Protocol V (DMSO induced differentiation as single cell culture)

1. Resuspend cells into EG medium at a concentration of 3×10^4 cells/ml, and plate into gelatinized tissue culture dishes. Culture for two days allowing cells attach and grow.

2. Change to RA differentiation medium (DMEM supplemented with 1% dimethyl sulfoxide (DMSO), 10% FBS, L-Glutamine, non-essential a.a., Penicillin-Streptomycin) and replace daily.

3. After 2 days, change to standard medium and replace daily.

Protocol VI (RA induced differentiation as single cell culture)

1. Resuspend cells into EG medium at a concentration of 3×10^4 cells/ml, and plate into gelatinized tissue culture dishes.
2. After two days, change to RA differentiation medium (DMEM supplemented with $0.3 \mu\text{M}$ all-trans retinoic acid, 10% FBS, L-Glutamine, Peniciline-Streptomycin) and replace daily.
2. After 2 days, change to standard medium and replace daily.

In vivo differentiation

1. Harvest EG culture and wash three times with PBS.
2. Count cells and pellet/resuspend them into a concentration of 2×10^6 cells/ml in PBS.
3. Inject 1 ml cells subcutaneously into nude mice, three mice per cell line.
4. After 3-4 weeks, dissect out tumor and washed with PBS twice. Cut tumor into 2-3 pieces and fix in 4% neutral Formalin more than 1 day. Fixed tissue blocks are processed for histology. Sections are stained with hematoxylin and eosin.

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4. Derivation, maintenance, and *in vitro* differentiation of EG cell lines: 8.5 d.p.c. embryos, resulted from crosses between male CAST/Ei (Jackson Lab, 7-8 week old) and female 129/SvEv (Taconic Farms, 7-8 week old) mice, were dissected according to Buehr and McLaren (31). To derive EG cell lines, we primarily followed Resnick, J.L. et al. (32) and Matsui, Y. et al. (32) with minor modifications: Primary cultures were carried out in EG culture medium (DMEM with 4.5 g/L glucose, 15% FBS, 100 units/ml penicillin-streptomycin, 2 mM L-glutamine, 0.01 mM non-essential amino acids, and 0.1 mM β -mercaptoethanol) supplemented with leukemia inhibitory factor (LIF, 1000 units/ml), basic fibroblast growth factor (bFGF, 1 ng/ml) and murine stem cell factor (SCF, 60 ng/ml). Cultures were trypsinized after nine days and replated in EG culture medium without bFGF and SCF supplementation. Colonies were picked, and individual EG cell lines were propagated on irradiated STO feeder layers in EG medium with LIF (1000 unit/ml). Spontaneous differentiation of EG cells on plastic was performed according to Matsui, Y. et al. (32). Differentiation using RA, DMSO and methylcellulose medium was carried out as described (33).

5. pEGFP-N3 vector (Clontech) was transfected into SJEG-1 cells by electroporation (250 μ F, 0.2 kV). Clones with stable integration, such as SJEG-1/GFP18-1, were obtained by G418 selection (500 μ g/ml). 8 to 12 cells were injected into C57BL/6 blastocysts. The injected embryos were transferred to pseudopregnant CD-1/VAF female mice. A total of 87 blastocysts were injected and 4 living male chimeras were obtained. Chimeric mice were identified by the agouti coat color. Chimera 1-1 was mated with 3 female CD-1 mice, resulting in three separate litters of offspring, in which about 1/3 were derived from germline transmitted SJEG-1/GFP18-1 cells.
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20. A pluripotent human stem cell culture was derived from primordial germ cells obtained from the gonadal ridges and attached mesenteries of a 7-week post fertilization female embryo as described (17). Embryoid bodies that formed spontaneously in the presence of LIF were harvested then disaggregated by incubation in 1 mg/ml collagenase/dispase (Boehringer Mannheim) at 37 °C for 30 min. Monolayer cell cultures derived from these embryoid bodies were routinely grown in RPMI 1640 and passaged weekly by using 0.05 % trypsin/0.53 mM EDTA.
21. Analysis of IGF2 polymorphism and allele-specific expression was performed essentially as described (30). PCR was performed using [³²P]-ATP end-labeled primer, and the products were resolved on 5% denaturing polyacrylamide gels following Apa I digestion.
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SSCP assays were developed for each gene: Kvlqt1: PCR was performed using primer set mLQT1-108/208 crossing multiple introns. 2 µl of the PCR products was used for subsequent SSCP carried out in a 20-µl volume containing 1x PCR buffer (BRL), 1 mM MgCl₂, 0.2 mM dNTP, 0.5 mM unlabeled primer, 0.1 mM end-labeled primer, and 0.5 units of Taq polymerase. Primer set mLQT1-U/L2 spanning two introns was used for SSCP in which mLQT1-U was end-labeled with [³²P]-ATP. Reaction products were electrophoresed on 8% SSCP gels (8% bis-acrylamide, 5% glycerol, 0.25x TBE buffer, 4 °C) at 40W for 6 hr. Igf2: PCR was performed using primer set Igf2-U/L spanning an intron. 10 ng of gel-purified PCR product was used as the template for subsequent SSCP reactions conducted as described for Kvlqt1. Reaction products were electrophoresed on 5% SSCP gels at 6 watts for 10 hr. L23mrp: PCR was performed using primer set L23mrp-101/201 spanning an intron. SSCP were performed using primer pair L23mrp-102/201 with 2 µCi of [α-³²P]-dATP added to each reaction. SSCP gels were run in the same manner as for Kvlqt1. Sequences of primers used were as follows: mLQT1-108, 5'-CCA CCA TCA AGG TCA TCA GGC GCA TGC-3' (SEQ ID NO: 1); mLQT1-208, 5'-GAG CTC CTT CAG GAA CCC TCA TCA GGG-3' (SEQ ID NO: 2); mLQT1-U, 5'-TTT GTT CAT CCC CAT CTC AG-3' (SEQ ID NO: 3); mLQT1-L2, 5'-TTG TTC GAT GGT GGG CAG G-3' (SEQ ID NO: 4); Igf2-U, 5'-GAC GTG TCT ACC TCT CAG GCC GTA CTT-3' (SEQ ID NO: 5); Igf2-L, 5'-GGG TGT CAA TTG GGT TGT TTA GAG CCA-3' (SEQ ID NO: 6); Igf2-U1, 5'-GAT CTC TCT GCT CCA CTT CC-3' (SEQ ID NO: 7).

7); Igf2-L1, 5'-TTG TTT AGA GCC AAT CAA AT-3'(SEQ ID NO: 8); Igf2r-U, 5'-CTG GAG GTG ATG AGT GTA GCT CTG GC-3'(SEQ ID NO: 9); Igf2r-L, 5'-GAG TGA CGA GCC AAC ACA GAC AGG TC-3'(SEQ ID NO:10); Igf2r-I2, 5'-CTC CTC TGC GGG GCC ATC-3'(SEQ ID NO: 11); H19-U, 5'-CCA CTA CAC TAC CTG CCT CAG AAT CTG C-3'(SEQ ID NO: 12); H19-L2, 5'-GGA ACT GCT TCC AGA CTA GG-3' (SEQ ID NO: 13); H19-L1, 5'-ACG GAG ATG GAC GAC AGG TG-3'(SEQ ID NO: 14); Snrpn-U, 5'-TGC TGC TGT TGC TGC TAC TG-3'(SEQ ID NO: 15); Snrpn-L, 5'-GCA GTA AGA GGG GTC AAA AGC-3'(SEQ ID NO: 16); Snrpn-I2, 5'-GCA GGT ACA CAA TTT CAC AAG AAG CAT T-3'(SEQ ID NO:17).

27. Quantitative sequencing assay: PCR was performed with primer set H19-U/L2 crossing an intron. Gel-purified PCR products were used in the subsequent sequencing reaction with primer H19-L1. Two methods of sequencing were used and shown to be concordant: (1) fluorescence-based automatic sequencing; (2) cycle sequencing reactions using the AmpliCycle sequencing kit and the provided protocol (Perkin Elmer). Reaction products were run on 7% sequencing gels at 90 W for 80 min and quantified on a PhosphorImager, with genomic DNA as a control for allele intensity.

28. SNUPE assays: Single nucleotide primer extension was performed as described (35) with minor modifications. Snrpn: PCR was performed with primer set Snrpn-U/L crossing an intron. SNUPE were performed using primer Snrpn-I2, and reaction products were resolved on 15% denaturing polyacrylamide gels. Igf2r: PCR was performed with primer set Igf2r-U/L crossing an intron. SNUPE was performed using primer Igf2r-I2 as described above.

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CLAIMS

1. A method of forming embryonic germ cells useful as a model system for studying imprinting, comprising:
 - mating a male and a female mammal of the same species to form a pregnant female mammal, wherein the male and the female mammals are sufficiently genetically divergent such that at least 50% of genes in resulting offspring have at least one sequence difference between alleles of said genes;
 - obtaining an embryo from the pregnant female mammal at a stage of embryonic development between when 2-3 somites become visualizable and when gonads are recognizable;
 - dissecting said embryo, dissociating cells of said embryo, and culturing the dissociated cells to provide embryonic germ cell lines.
2. The method of claim 1 wherein the mammals are mice.
3. The method of claim 2 wherein the embryo is obtained at day 7-10 post conception.
4. The method of claim 1 wherein the female mammal is a 129/SvEv mouse.
5. The method of claim 1 wherein the male mammal is a CAST/Ei mouse.
6. The method of claim 1 wherein the dissociated cells are cultured on a feeder cell layer.
7. The method of claim 1 wherein the posterior third of the embryo is dissected and used to form dissociated cells.
8. The method of claim 1 wherein the genital ridge of the embryo is dissected out and used to form dissociated cells.
9. The method of claim 1 wherein gonads of the embryo are dissected out and used to form dissociated cells.
10. The method of claim 1 wherein the wherein the male and the female mammals are sufficiently genetically divergent such that at least 60% of genes in resulting offspring have at least one sequence difference between alleles of said genes.
11. The method of claim 1 wherein the wherein the male and the female mammals are sufficiently genetically divergent such that at least 75% of genes

- in resulting offspring have at least one sequence difference between alleles of said genes.
12. The method of claim 1 wherein the wherein the male and the female mammals are sufficiently genetically divergent such that at least 90% of genes have at least one sequence difference between alleles of said genes
 13. The method of claim 1 wherein the wherein the male and the female mammals are sufficiently genetically divergent such that at least 95% of genes in resulting offspring have at least one sequence difference between alleles of said genes.
 14. A method of inducing imprinting *in vitro*, comprising:
culturing mammalian embryonic germ cells in suspension culture under conditions in which the embryonic germ cells differentiate, whereby expression of one or more imprintable genes changes from approximately equal biallelic to preferentially uniparental.
 15. The method of claim 14 wherein the germ cells are grown on plastic in the absence of feeder cells.
 16. The method of claim 14 wherein the germ cells are grown in the presence of dimethylsulfoxide.
 17. The method of claim 14 wherein the germ cells are grown in the presence of retinoic acid.
 18. The method of claim 14 wherein the germ cells are grown on a methyl-cellulose containing medium.
 19. The method of claim 14 wherein the germ cells contain a selectable marker under transcriptional control of a tissue-specific promoter, and the germ cells are subjected to selection conditions to select for germ cells which have differentiated into a lineage which activates the tissue-specific promoter.
 20. The method of claim 14 wherein the germ cells form an embryoid body.
 21. A method of inducing imprinting *in vivo*, comprising:
injecting one or more mammalian embryonic germ cells into a nude mouse, whereby the embryonic germ cells differentiate and form a teratocarcinoma and whereby expression of one or more imprintable genes changes from approximately equal biallelic to preferentially uniparental.
 22. A method of inducing imprinting *in vivo*, comprising:

injecting a mammalian embryonic germ cell into a blastocyst of a mammal;

implanting the blastocyst into a pseudopregnant mammal so that the blastocyst develops into a chimeric mammal, whereby expression of one or more imprintable genes in somatic cells derived from the embryonic germ cell becomes preferentially uniparental.

23. The method of claim 22 wherein the mammalian embryonic germ cell is transfected with a vector which expresses a detectable marker protein, prior to the step of injecting.

24. An isolated and purified mammalian embryonic germ cell line which:
expresses one or more imprintable genes in a biparental fashion;
forms cells which express one or more imprintable genes in a biparental manner;

differentiates to form cells which express said one or more imprintable genes in a preferentially uniparental fashion.

25. The isolated and purified mammalian embryonic germ cell line of claim 24 which is a mouse cell line.

26. The isolated and purified mammalian embryonic germ cell line of claim 24 which differentiates *in vitro*.

27. The isolated and purified mammalian embryonic germ cell line of claim 24 which differentiates *in vivo*.

28. The isolated and purified mammalian embryonic germ cell line of claim 24 which imprints *in vitro*.

29. The isolated and purified mammalian embryonic germ cell line of claim 24 which imprints *in vivo*.

30. A method of testing substances as candidate drugs comprising:
contacting the isolated and purified mammalian embryonic germ cell line of claim 24 with a test substance;

assaying imprinting of one or more imprintable genes.

31. The method of claim 30 further comprising the step of:

identifying a test substance as a candidate drug for treating cancer if the test substance enhances imprinting of a gene whose imprinting is lost in cancer, or if the test substance inhibits imprinting of a gene whose imprinting is gained in cancer.

32. The method of claim 30 wherein differentiation of the mammalian embryonic germ cell line is induced before, after, or during the step of contacting.
33. The method of claim 30 wherein the mammalian embryonic germ cell line is transfected with a vector encoding a marker protein, the mammalian embryonic germ cell line is injected into a blastocyst, and the blastocyst is implanted in a pseudopregnant female.
34. The method of claim 30 wherein the step of assaying is done by single strand conformation polymorphism analysis.
35. The method of claim 30 wherein the step of assaying is done by quantitative sequencing.
36. The method of claim 30 wherein the step of assaying is done by single nucleotide primer extension.
37. The method of claim 30 wherein the step of assaying is done by hot stop PCR.
38. A method of testing substances as candidates drugs comprising:
 - contacting the isolated and purified mammalian embryonic germ cell line of claim 24 with a test substance;
 - assaying methylation of one or more imprintable genes.
39. The method of claim 38 further comprising the step of:
 - identifying a test substance as a candidate drug for treating cancer if the test substance enhances methylation of a gene whose methylation is lost in cancer, or if the test substance inhibits methylation of a gene whose methylation is gained in cancer.
40. A method of making a chimeric animal which can be used as a model system for imprinting, comprising:
 - transfecting a mammalian embryonic germ cell with a vector which expresses a detectable marker protein, wherein the embryonic germ cell expresses one or more imprintable genes in a biparental manner;
 - injecting the transfected mammalian embryonic germ cells into a blastocyst of a mammal;
 - implanting the blastocyst into a pseudopregnant mammal, whereby the blastocyst develops into a chimeric mammal, wherein the chimeric mammal

expresses the one or more imprintable genes in a preferentially uniparental fashion.

41. A chimeric mammal made by the process of claim 40.
42. The method of claim 30 wherein post-translational modification of histones is determined.
43. The method of claim 31 wherein post-translational modification of histones is determined.
44. The method of claim 32 wherein post-translational modification of histones is determined.
45. The method of claim 33 wherein post-translational modification of histones is determined.
46. A method for isolating methylated CpG islands comprising the steps of:
 - a. digesting eukaryotic genomic DNA with a first restriction endonuclease which recognizes a recognition sequence found in A/T rich regions of DNA or found in CpG island-poor regions of DNA;
 - b. digesting the eukaryotic genomic DNA with a second restriction endonuclease which recognizes a 4 base-pair sequence in unmethylated C/G rich regions;
 - c. isolating fragments of at least 1 kb formed by the step of digesting and inserting the fragments into bacterial vectors;
 - d. transforming non-methylating, non-restricting bacteria with the bacterial vectors to propagate the vectors and render the fragments' progeny unmethylated;
 - e. digesting the unmethylated fragments with a third restriction endonuclease which recognizes a sequence of at least 6 base pair in G/C rich regions;
 - f. isolating the resulting fragments and inserting said fragments into bacterial vectors to form a library of sequences which are enriched for sequences derived from methylated CpG islands in the eukaryotic genome.
47. The method of claim 46 further comprising the step of eliminating undesired repetitive elements by digesting the resulting fragments referred to in step (f)

- with a fourth restriction endonuclease which recognizes a unique site in the repetitive elements.
48. The method of claim 46 wherein the first restriction endonuclease is Mse I.
 49. The method of claim 46 wherein the second restriction endonuclease is Hpa II
 50. The method of claim 46 wherein the third restriction endonuclease is Eag I.
 51. The method of claim 46 wherein the fourth restriction endonuclease recognizes a site in element SVA.
 52. The method of claim 46 wherein the eukaryotic genomic DNA is isolated from a male.
 53. The method of claim 46 wherein the eukaryotic genomic DNA is isolated from a tumor.
 54. The method of claim 46 wherein the eukaryotic genomic DNA is isolated from a Wilm's tumor.
 55. The method of claim 46 further comprising the step of:
testing one or more members of the library of sequences which are enriched for sequences derived from methylated CpG islands to identify sequences which are differentially methylated between maternal and paternal chromosomes.
 56. The method of claim 46 further comprising the step of:
testing one or more members of the library of sequences which are enriched for sequences derived from methylated CpG islands to identify sequences which are differentially methylated between hydatidiform moles and teratomas.
 57. The method of claim 46 further comprising the step of:
mapping one or more members of the library of sequences to a genomic region, whereby location of a methylated CpG island is determined.
 58. The method of claim 57 further comprising the step of:
identifying an imprinted gene adjacent to the methylated CpG island;
identifying a disease which is preferentially transmitted by one parent and which is genetically linked to region of genomic DNA which contains the imprinted gene, whereby the imprinted gene is thereby indicated as a candidate gene involved in transmitting the disease.

59. The method of claim 46 further comprising the step of:

testing a population of individuals for methylation of a member of the library of sequences, whereby a sequence which is differentially methylated between individuals is a methylation polymorphism which can be used to identify individuals.

60. A library of fragments which are enriched at least 100-fold in methylated CpG islands relative to total genomic DNA.

61. The library of fragments of claim 60 which comprises at least 50 distinct members.

62. A method for testing substances as candidate drugs, comprising:

contacting a mouse made by the process of claim 21 with a test substance;

identifying a test substance as a candidate drug if it inhibits the growth of the teratoma or causes regression of the teratoma.

63. A method of providing an assessment of risk of developing cancer, comprising the steps of:

determining methylation status of a CpG island selected from the group identified in Table 2 in a sample of a patient;

comparing the methylation status of the CpG island to that found in a control group of healthy individuals;

identifying the patient as having an increased risk of developing cancer if methylation status of the CpG island is perturbed relative to the methylation status in the control group.

64. The method of claim 63 wherein the status of at least 5 CpG islands is determined and the patient is identified as having an increased risk if at least 3 of said CpG islands have perturbed methylation status relative to control group.

65. A method of providing diagnostic information relative to cancer, comprising the steps of:

determining methylation status of a CpG island selected from the group identified in Table 2 in a sample of a tissue of a patient suspected of being neoplastic;

comparing the methylation status of the CpG island to that found in a control sample of said tissue which is apparently normal;

identifying the patient as having an increased risk of developing cancer if methylation status of the CpG island is perturbed relative to the methylation status in the control sample.

66. The method of claim 65 wherein the status of at least 5 CpG islands is determined and the patient is identified as having an increased risk if at least 3 of said CpG islands have perturbed methylation status relative to control sample.
67. An isolated and purified methylated CpG island which is selected from those shown in Table 2.
68. The CpG island of claim 67 which retains its methylation pattern found in a human.
69. The CpG island of claim 68 wherein the methylation pattern found in a human is methylated in normal individuals, but not in diseased or disease-prone individuals.
70. The CpG island of claim 68 wherein the methylation pattern found in a human is unmethylated in normal individuals, but methylated in diseased or disease-prone individuals.
71. The CpG island of claim 68 wherein the methylation pattern found in a human is methylated in normal tissues, but not in diseased or diseased tissues.
72. The CpG island of claim 68 wherein the methylation pattern found in a human is unmethylated in normal tissues, but methylated in diseased tissues.
73. The CpG island of claim 67 which is devoid of its methylation pattern found in a human.
74. A method of identifying imprinted genes comprising the steps of:
 - identifying a gene which is within about 2 million base pairs of a CpG island identified in Table 2 in the human genome;
 - determining whether the gene is preferentially uniparentally expressed;
 - identifying the gene as an imprinted gene if it is preferentially uniparentally expressed.

75. An isolated and purified methylated CpG island which is methylated in both maternal and paternal alleles of a human.
76. The isolated and purified methylated CpG island of claim 75 wherein the human is healthy.
77. The isolated and purified methylated CpG island of claim 75 wherein the methylation is not associated with a disease state.
78. An isolated and purified methylated CpG island which is biallelically methylated in some humans and not biallelically methylated in other humans, thus comprising a methylation polymorphism.
79. The CpG island of claim 78 which is methylated in normal tissue of a human having a tumor but not in tumor tissue of the human.
80. The CpG island of claim 78 which is methylated in both normal and tumor tissue of a human who has a tumor.

CAST/Ei ♂ x ♀ 129/SvEv

Figure 1.

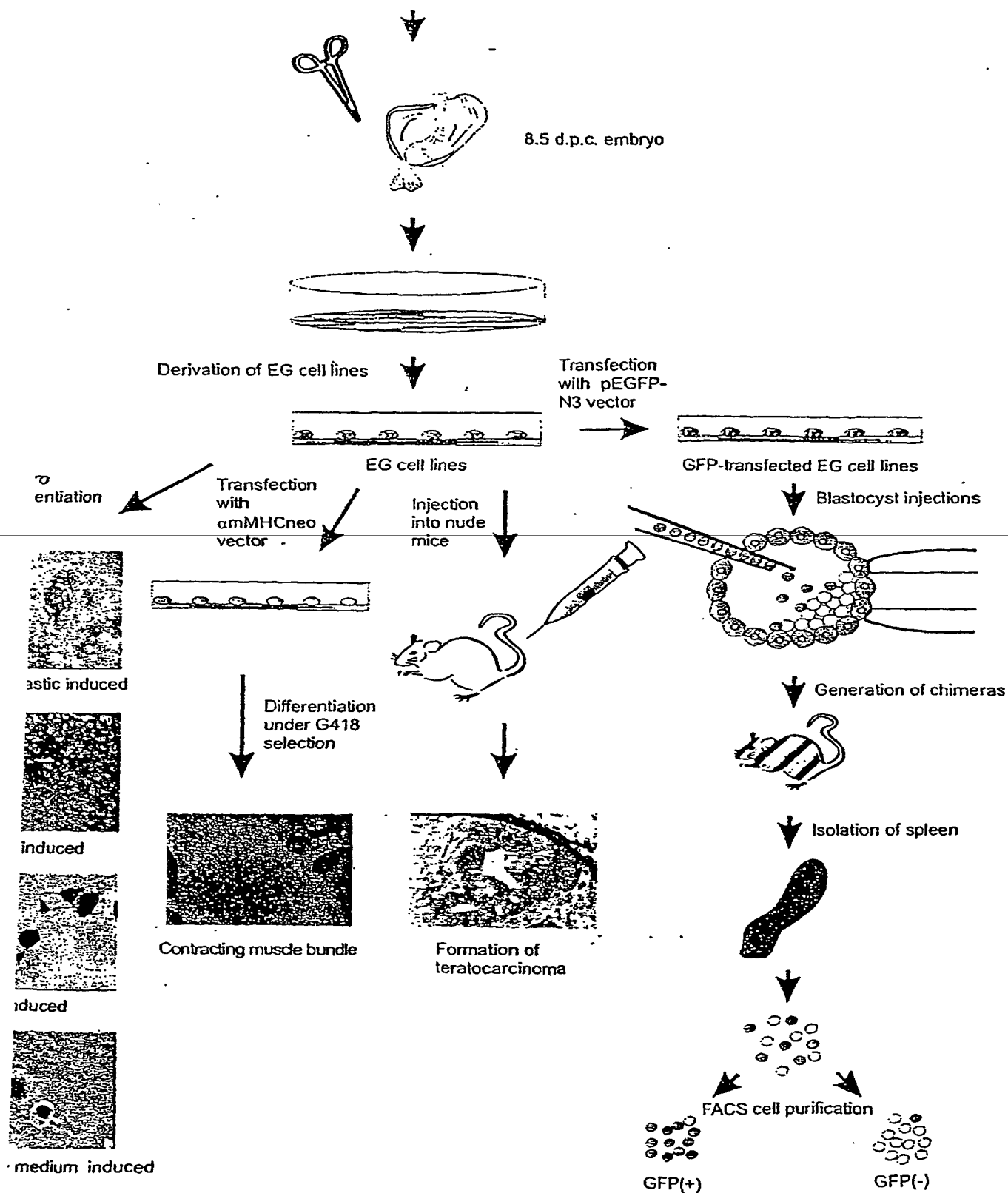
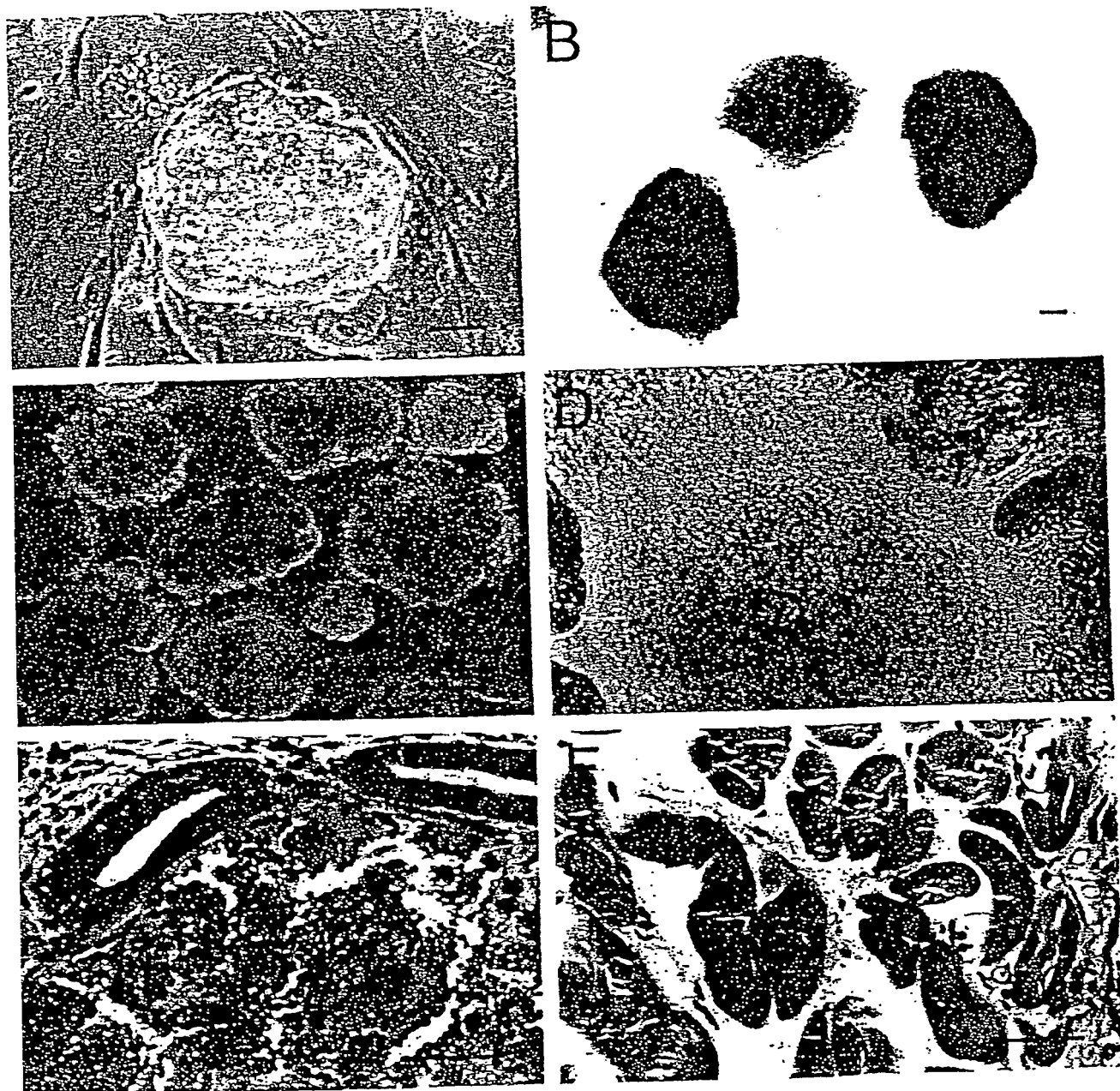


Figure 2.



A

Figure 3A.

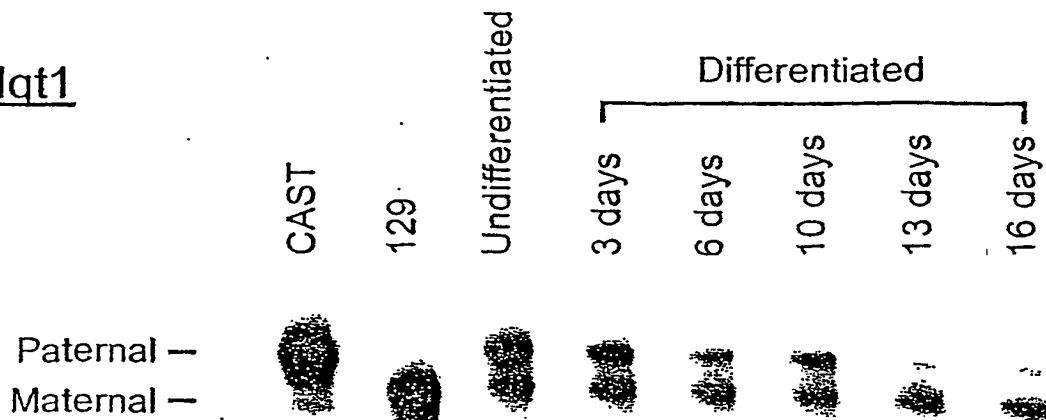
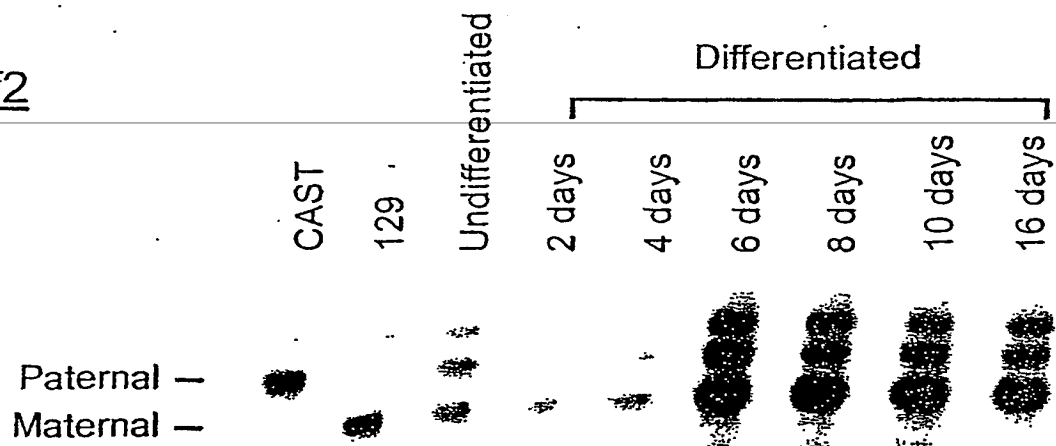
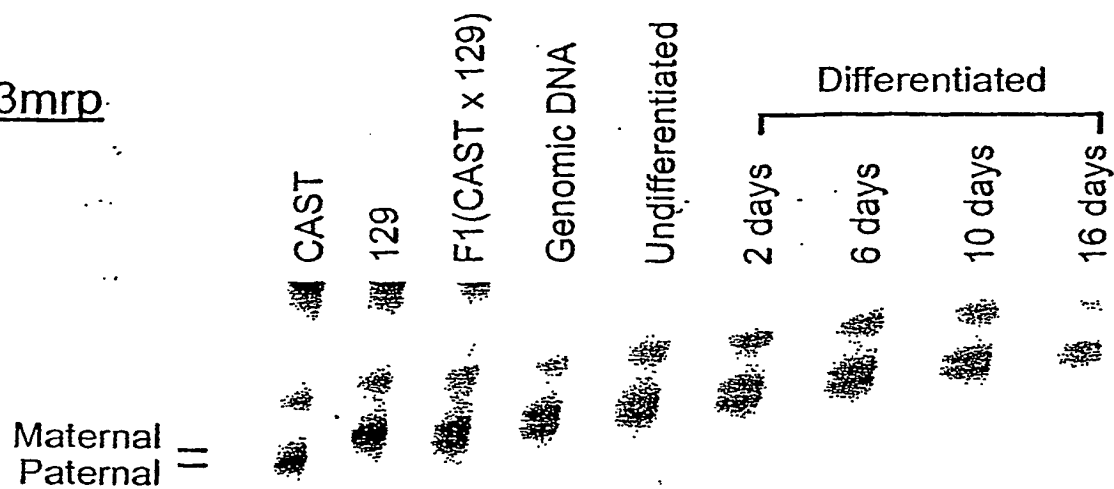
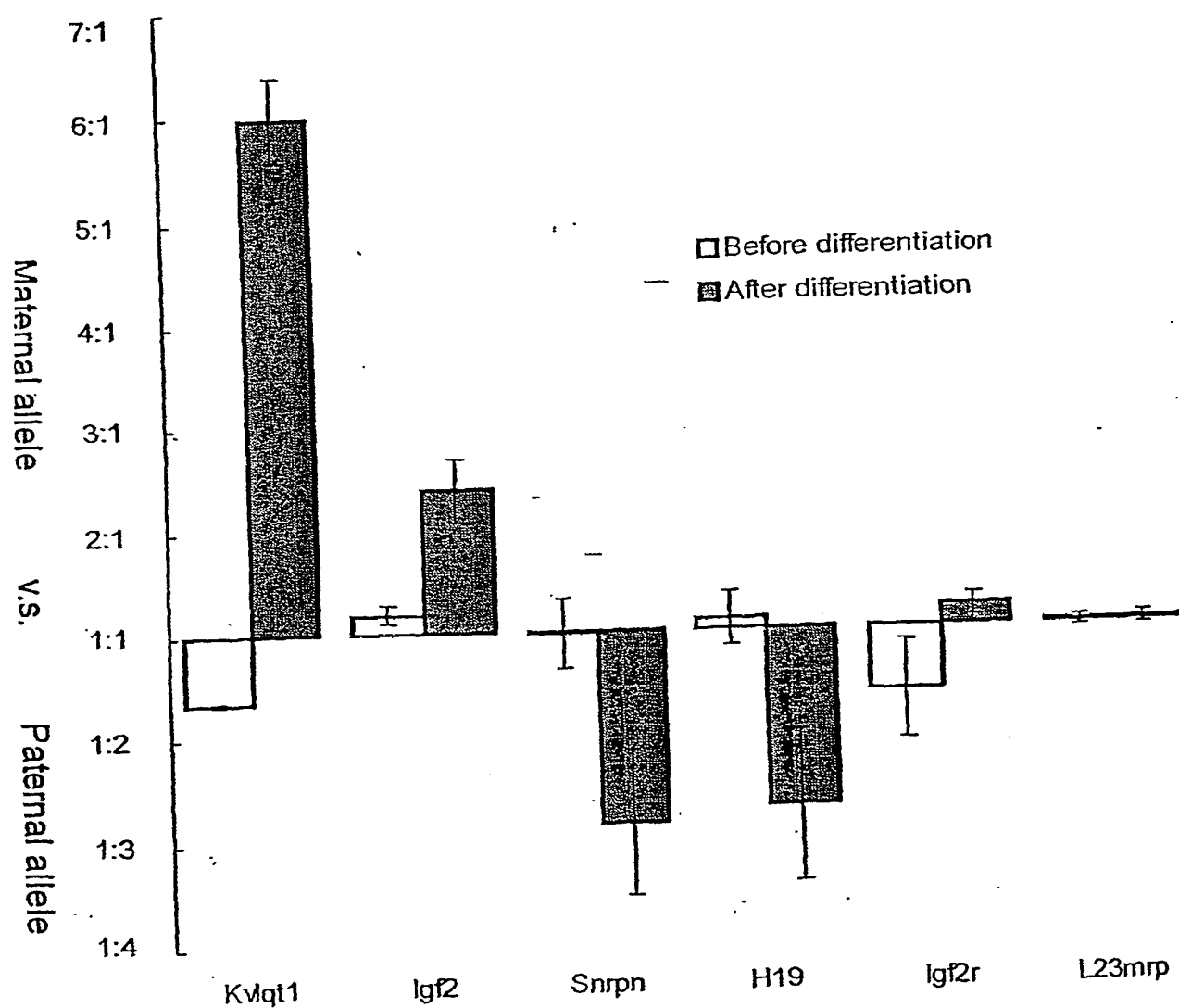
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Figure 3B.



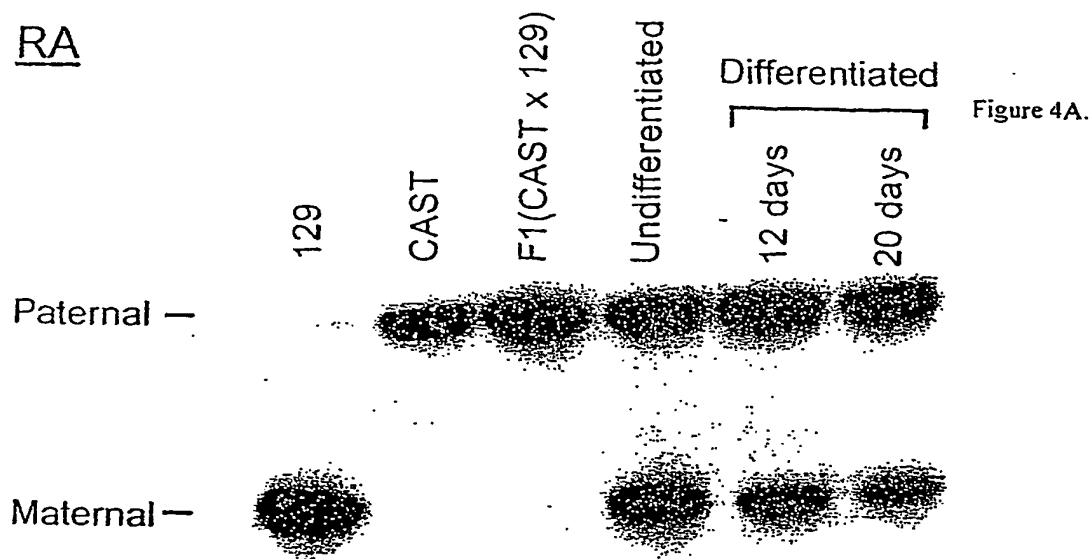
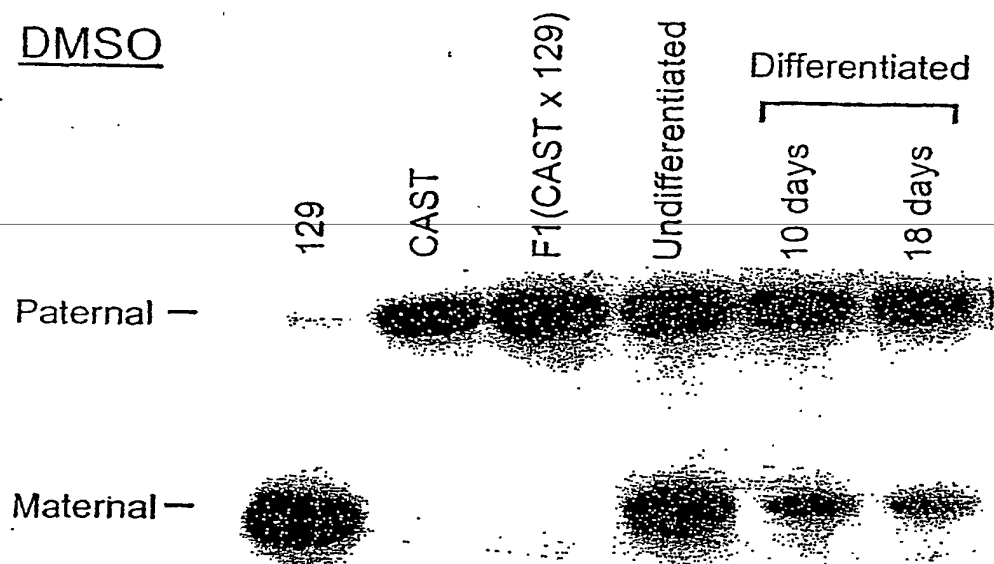
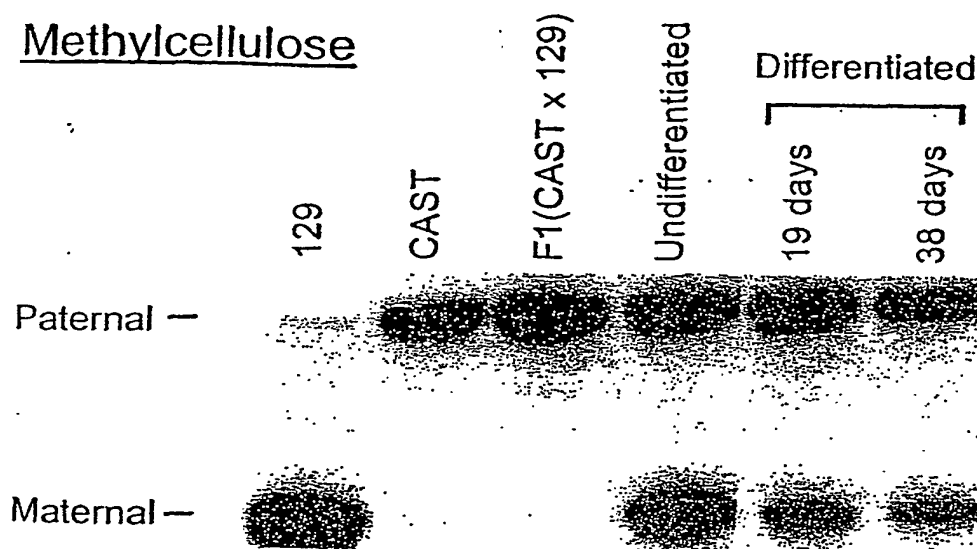
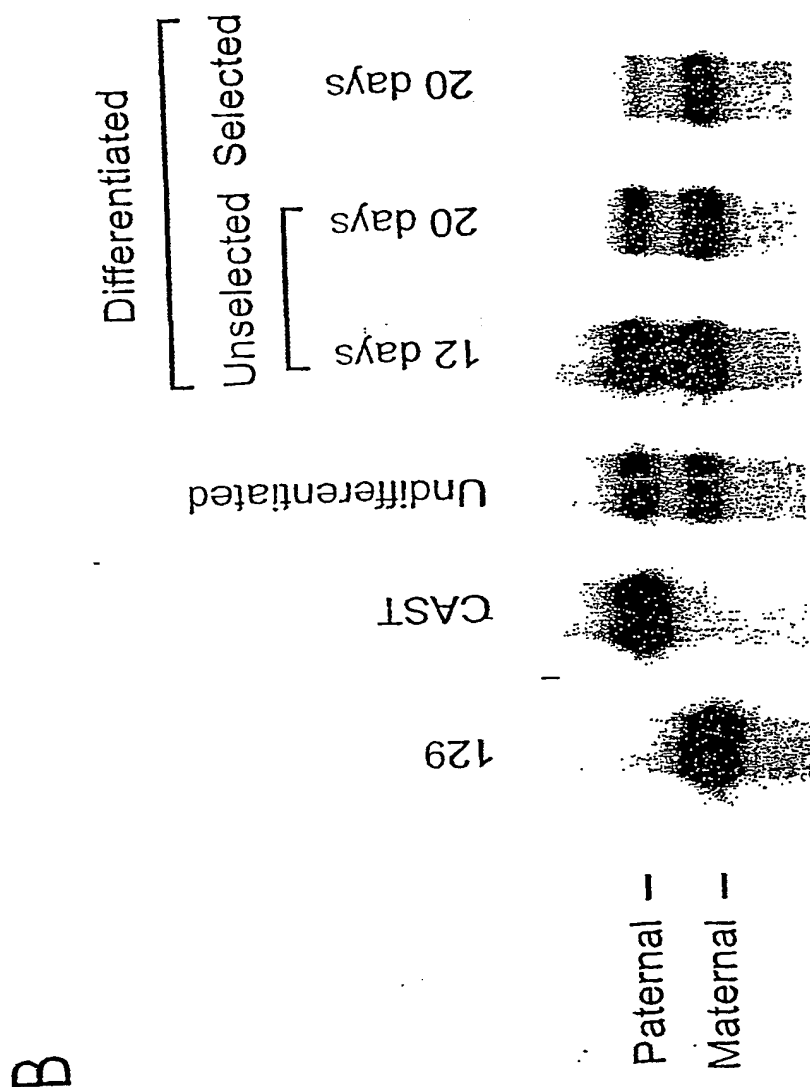
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Figure 4B.



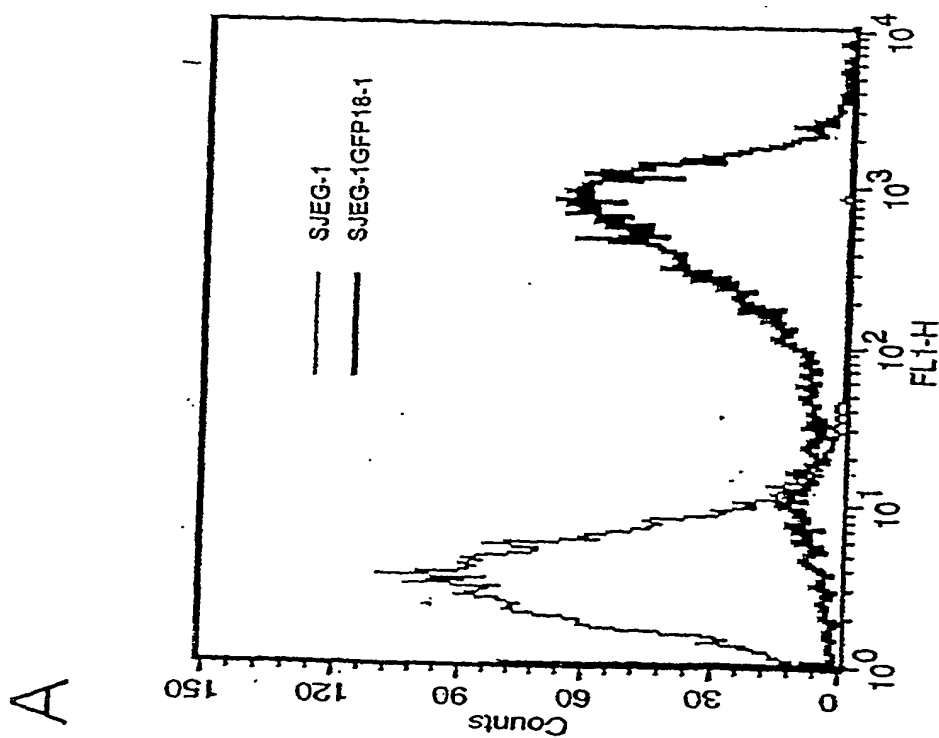
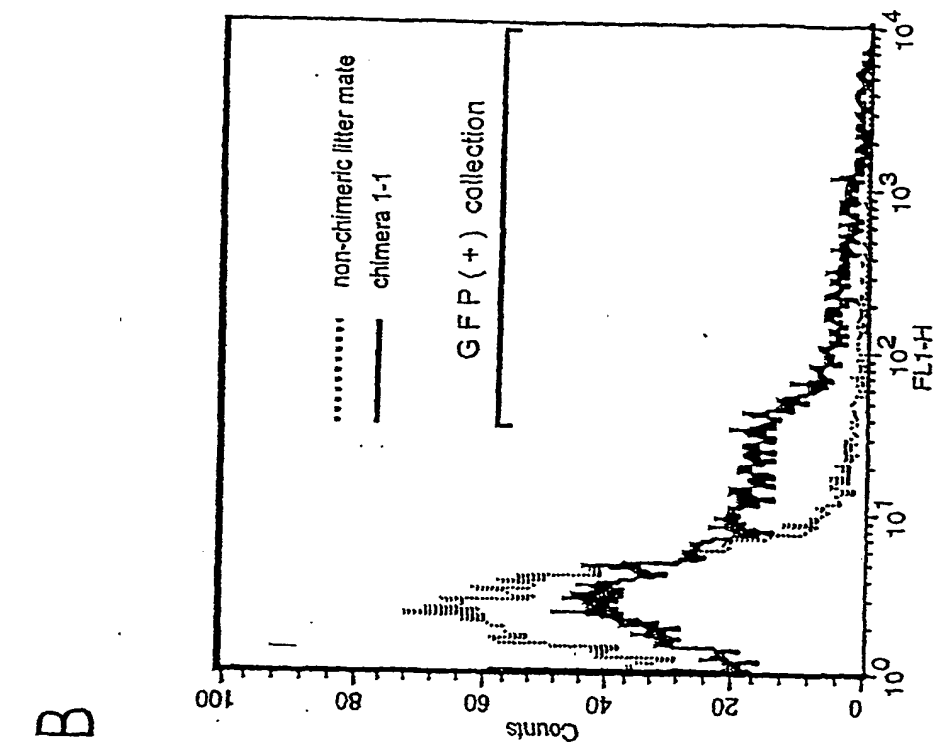


Figure 5A and B.

Figure 5C, D, and E.

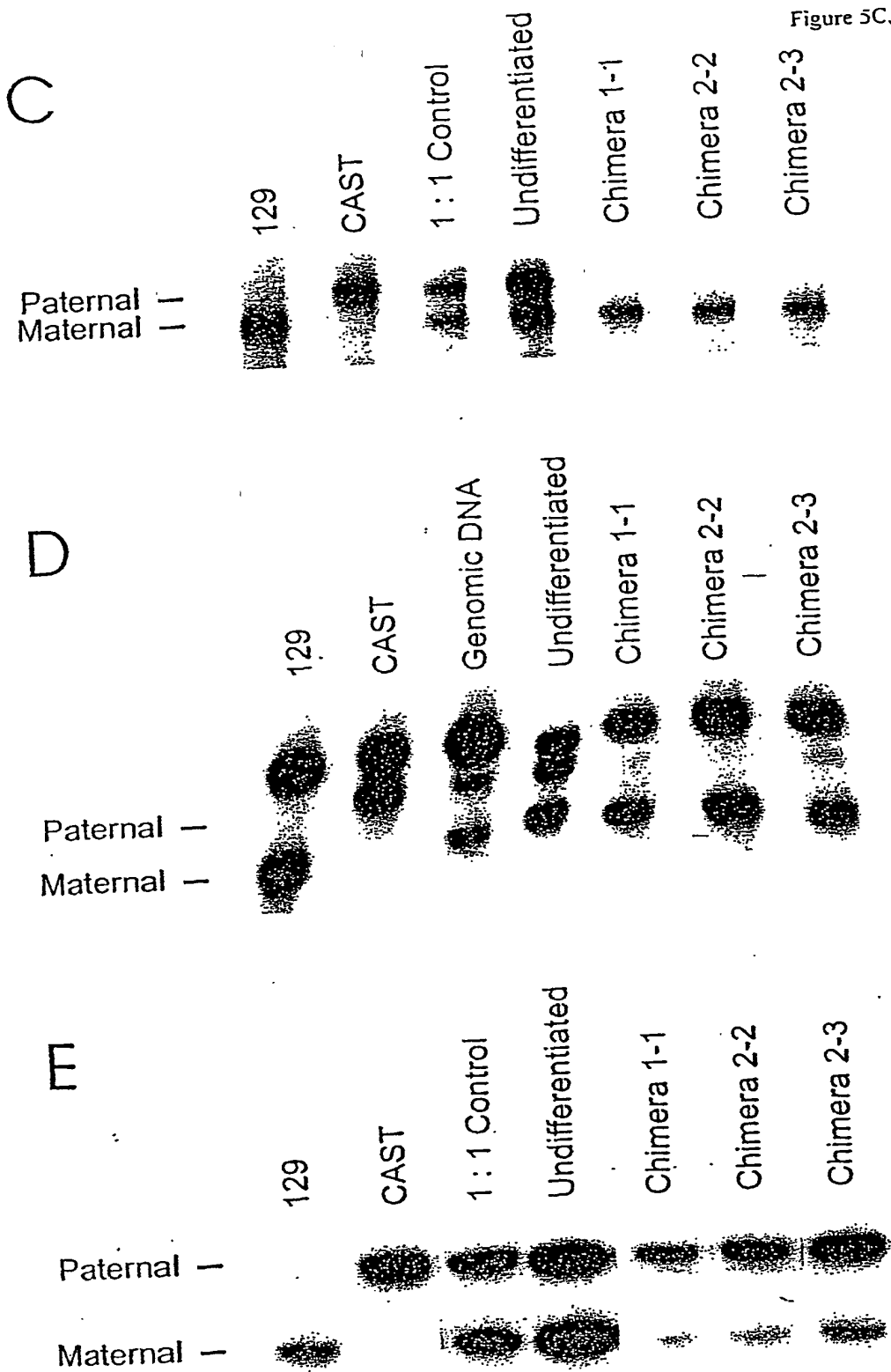


Figure 6A.

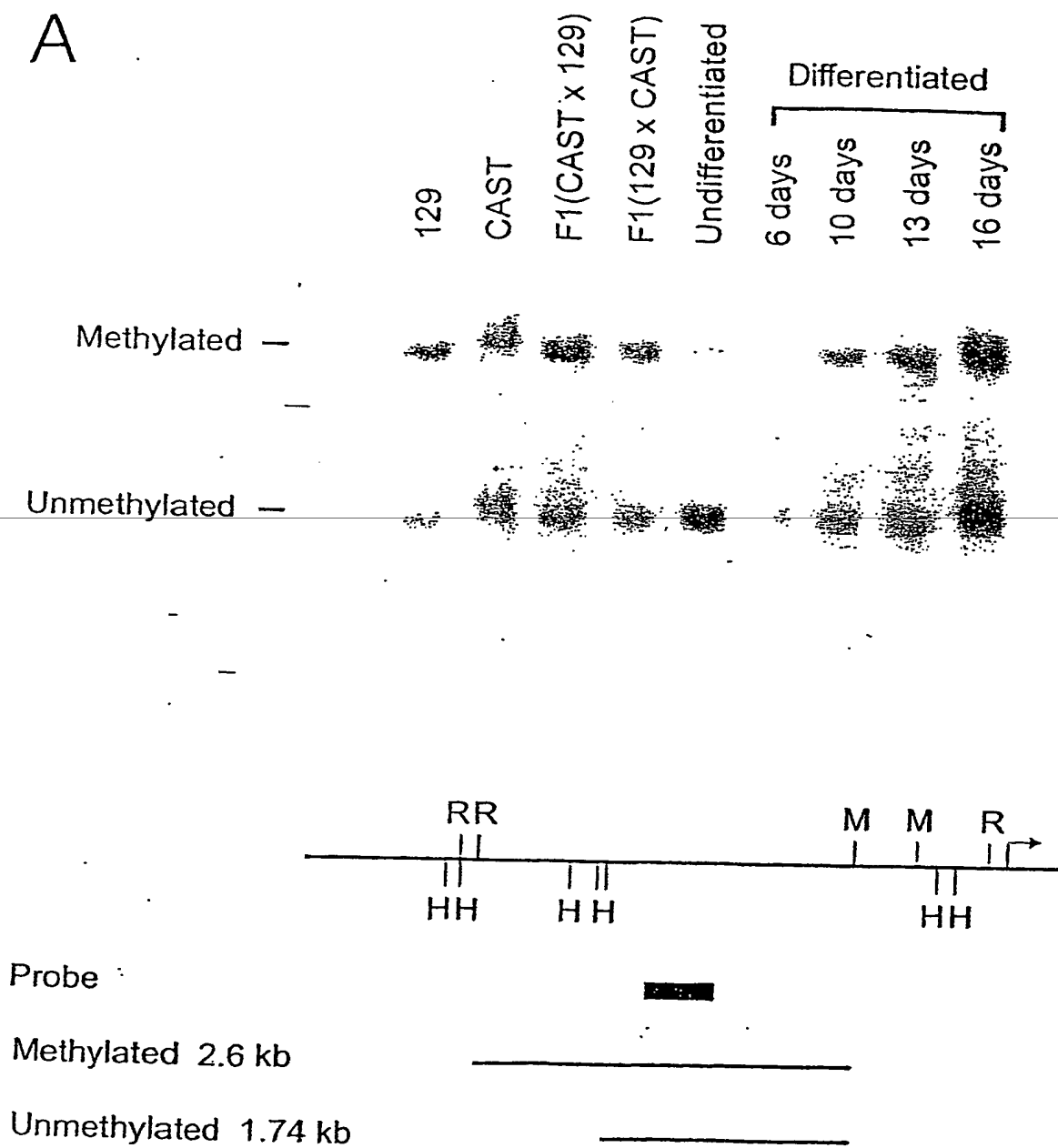


Figure 6B.

B

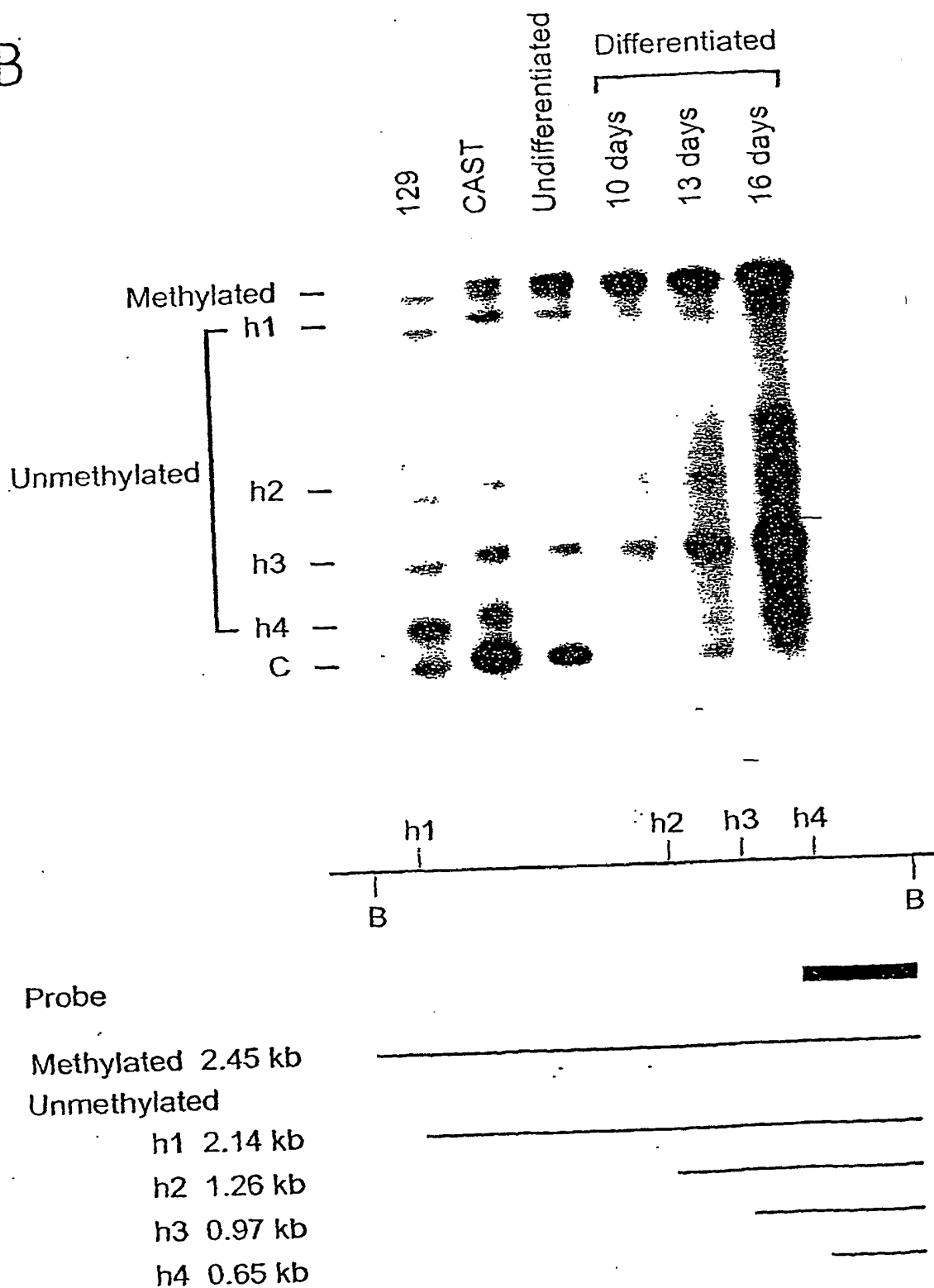
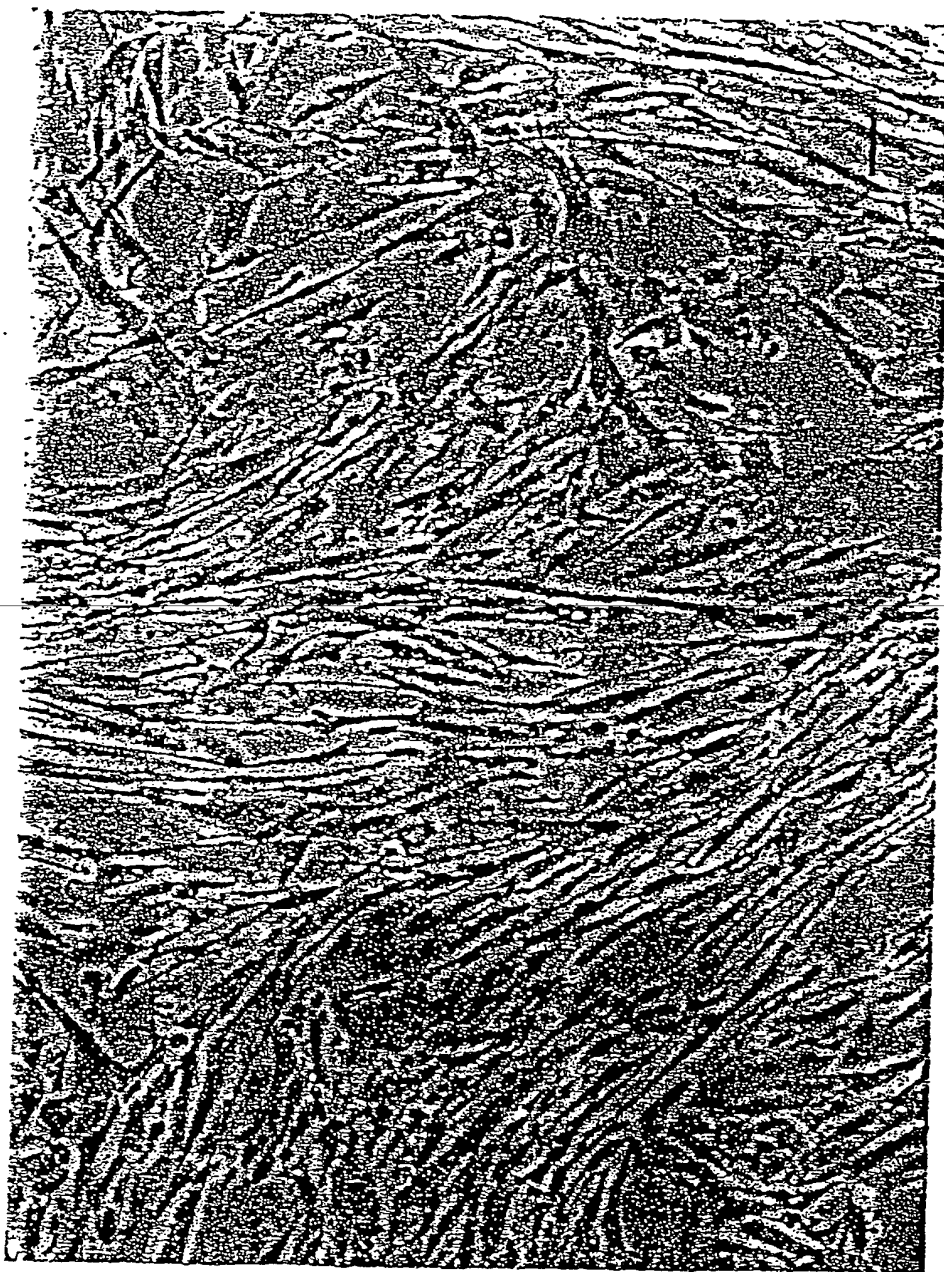


Figure 7A.



A

Figure 7B and C.

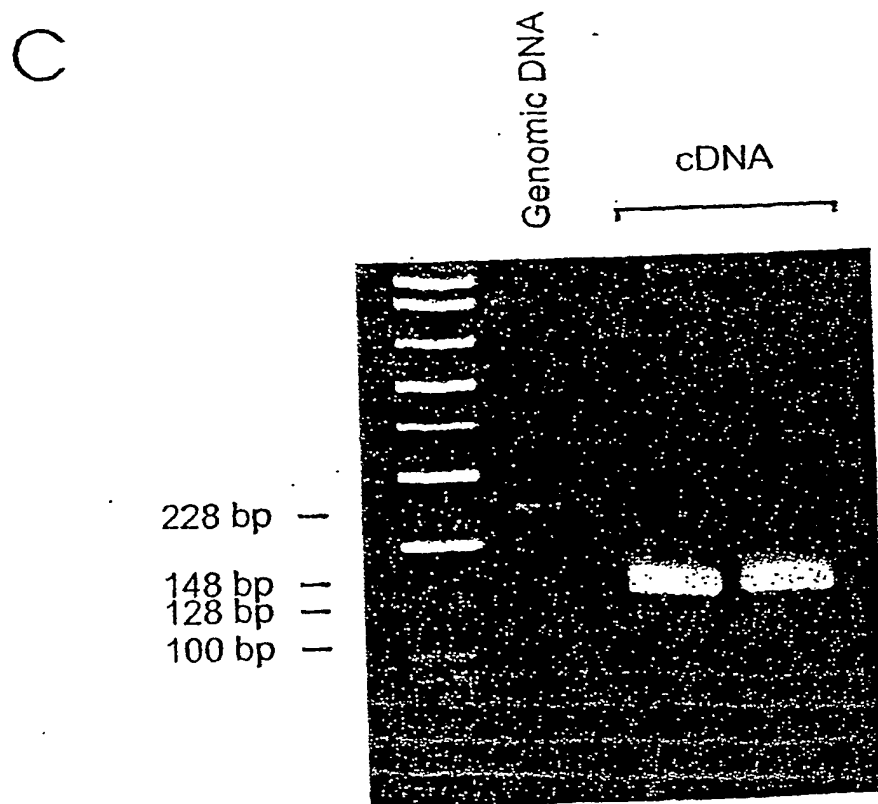
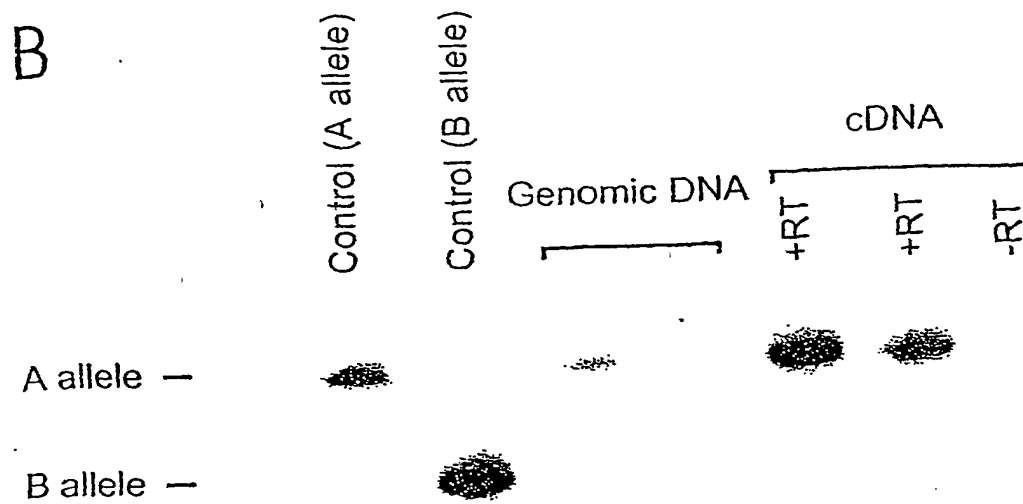


Figure 7D.

D

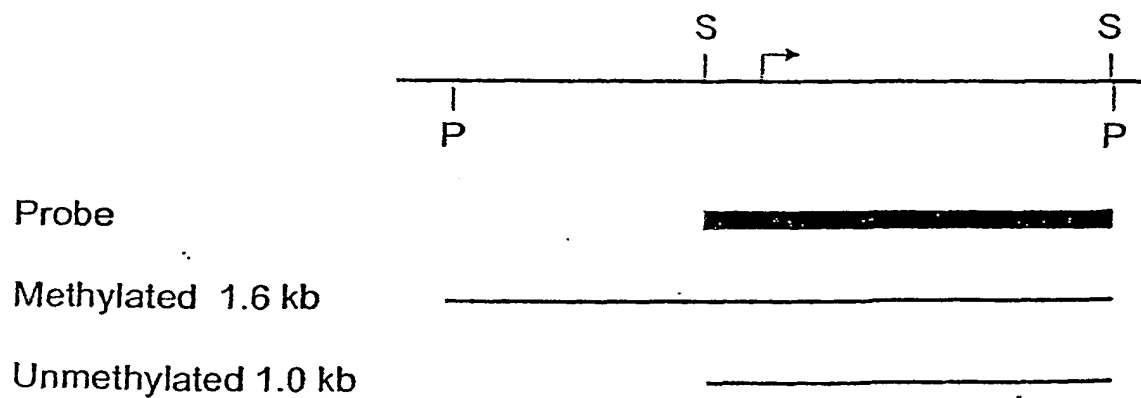
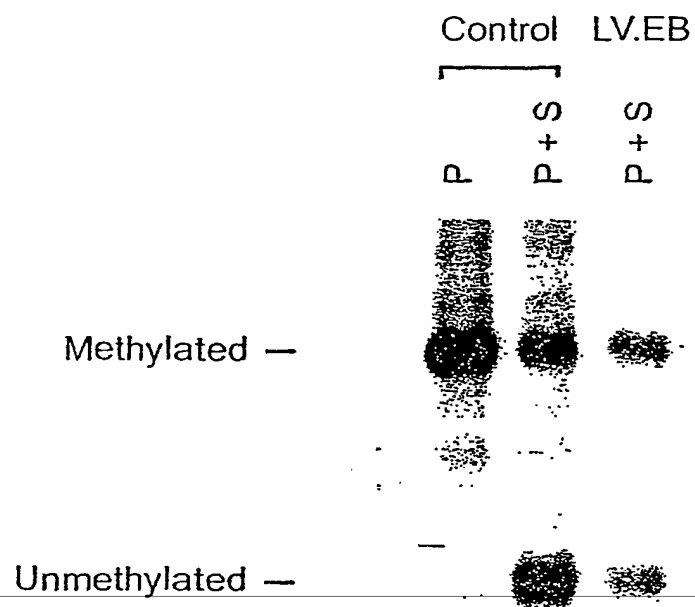


Figure 8.

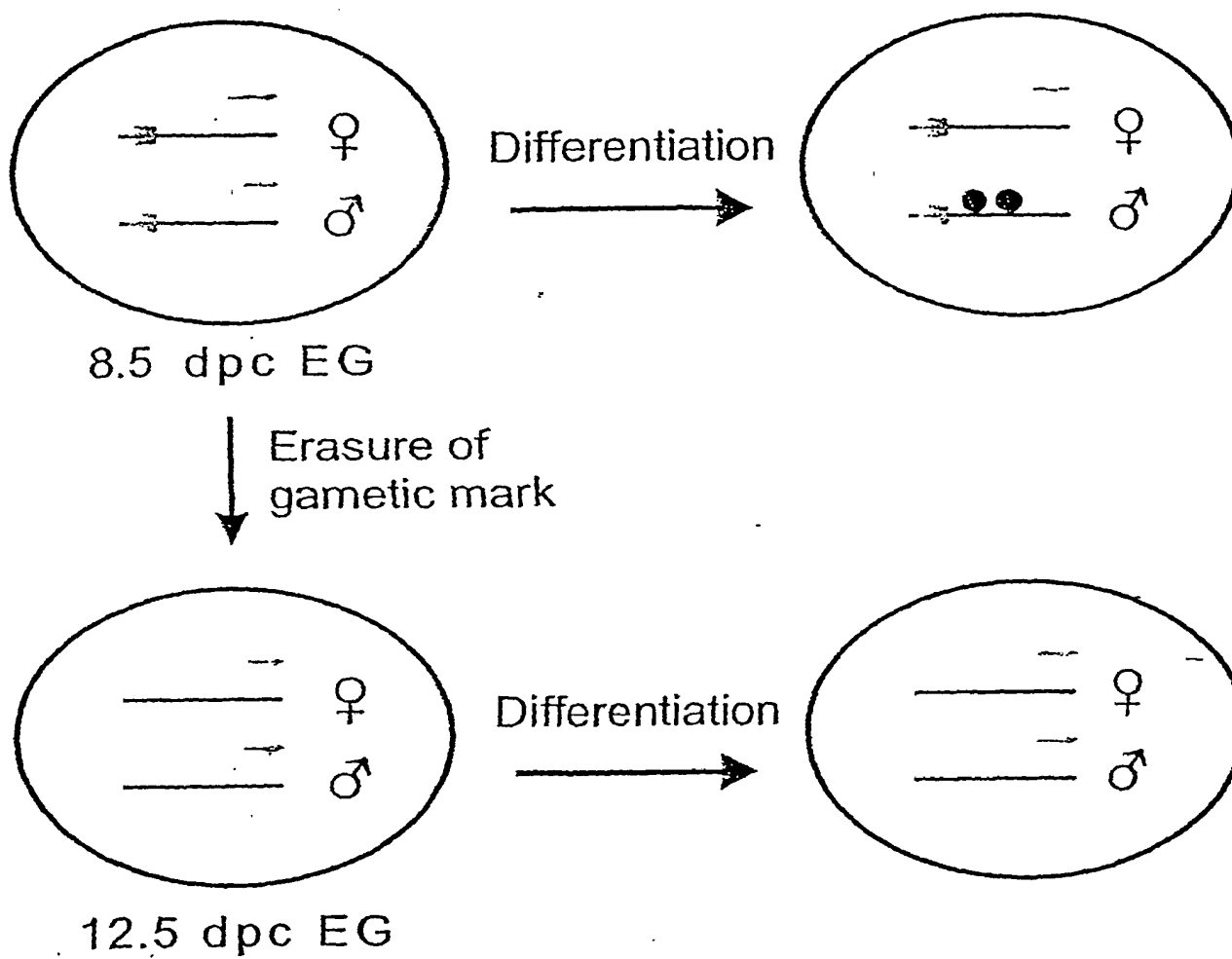
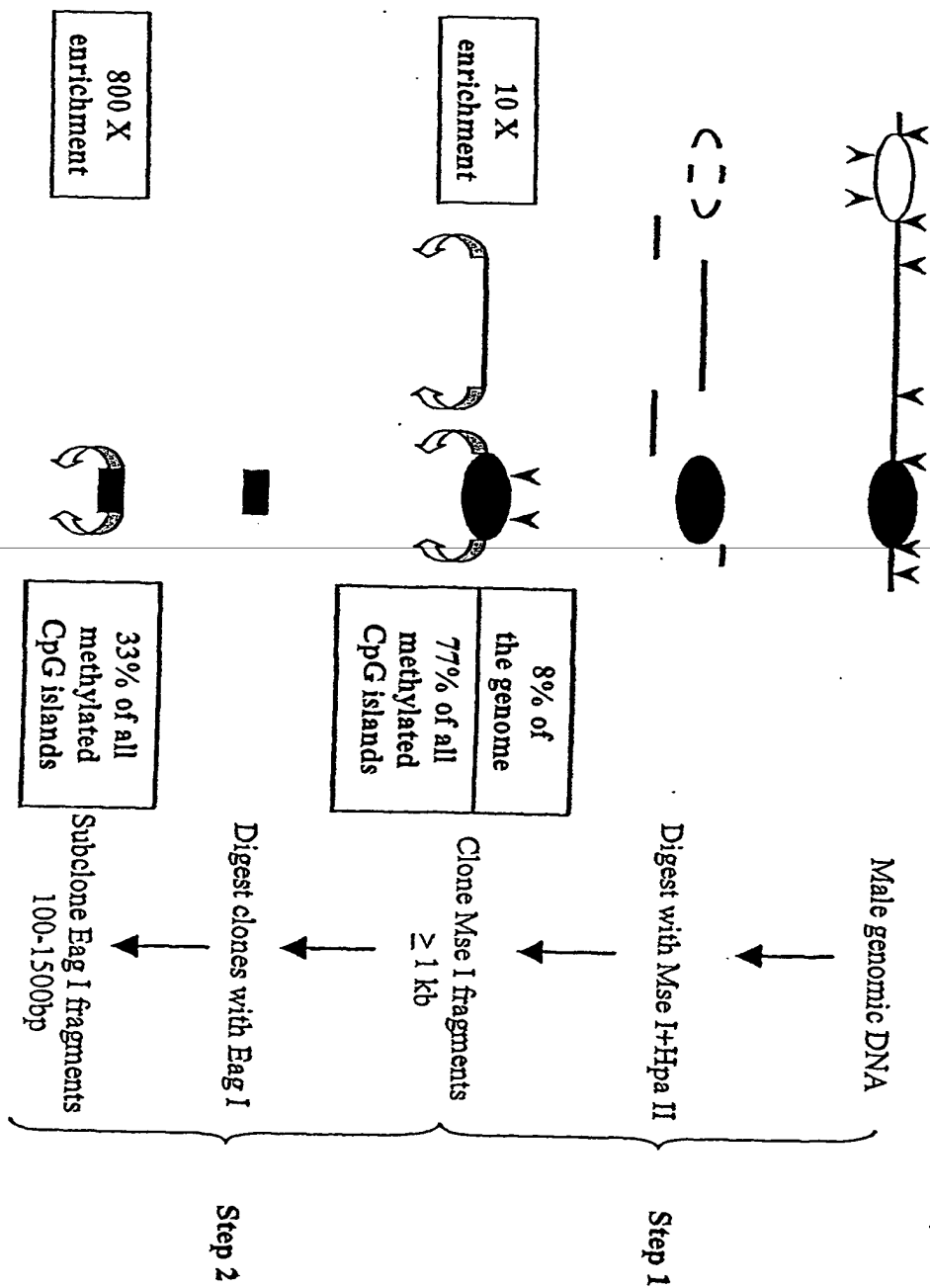


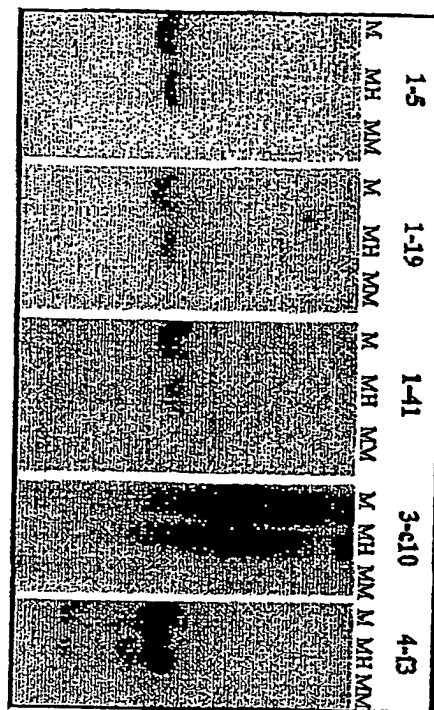
Fig. 9



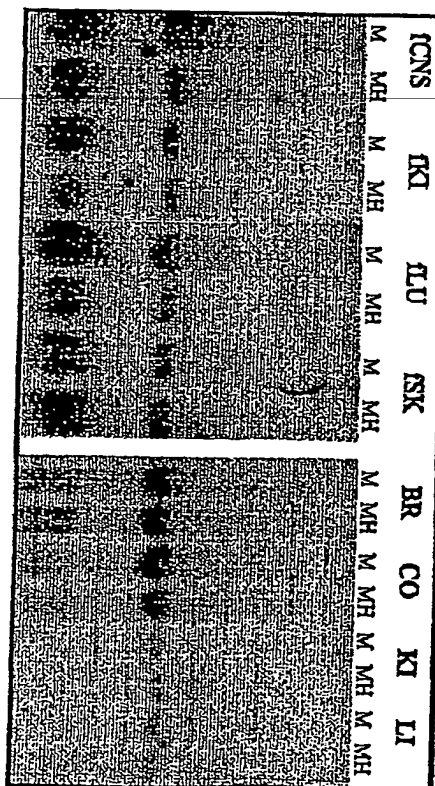
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Figure 10

A



B



C

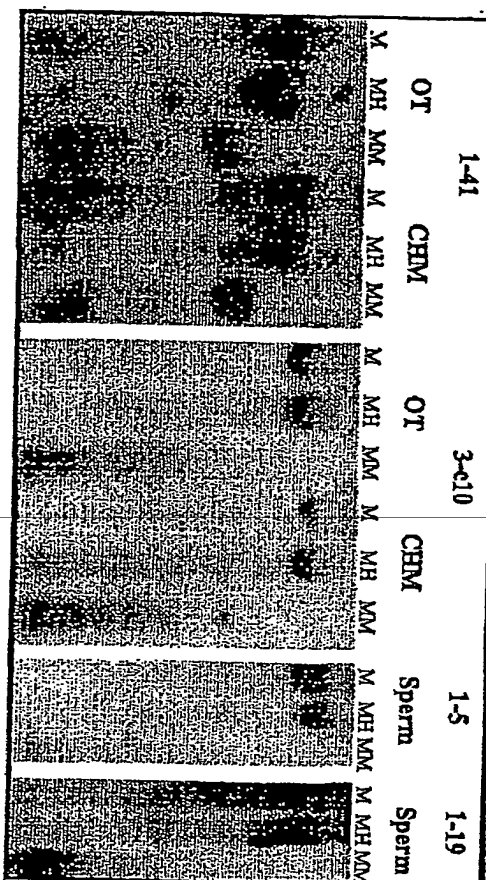


figure 11

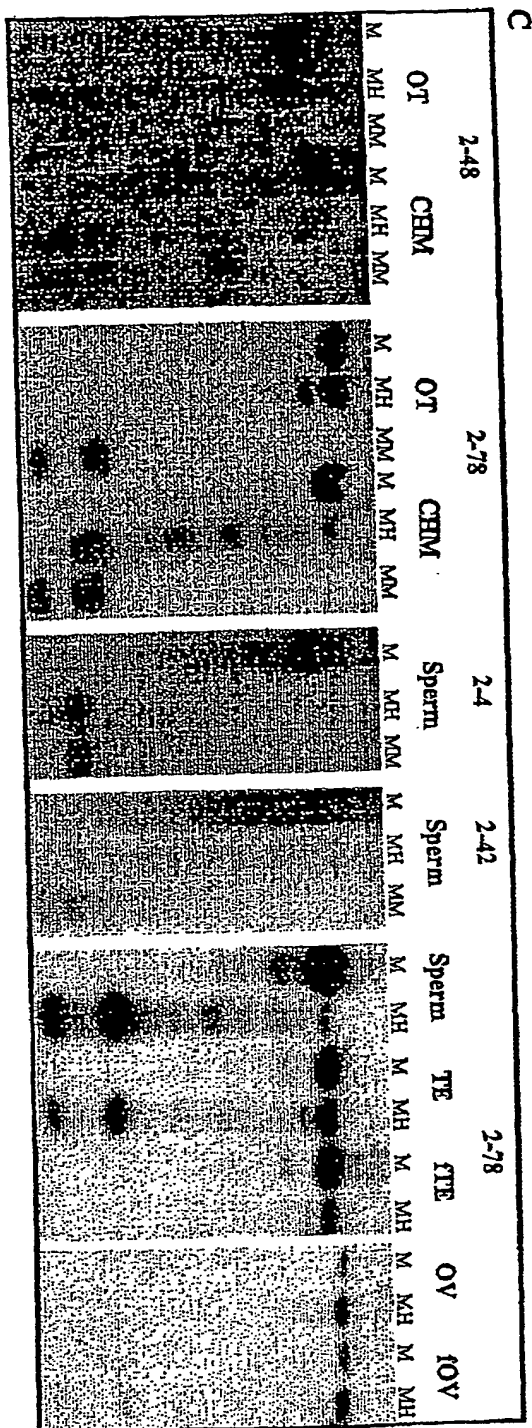
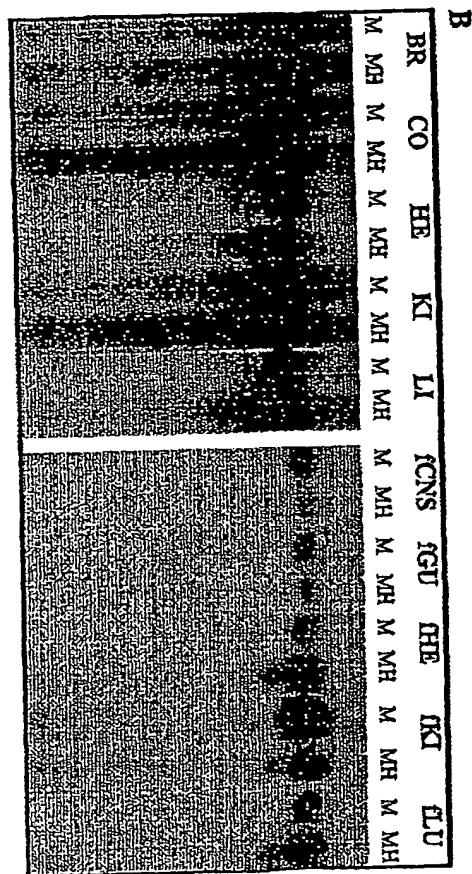
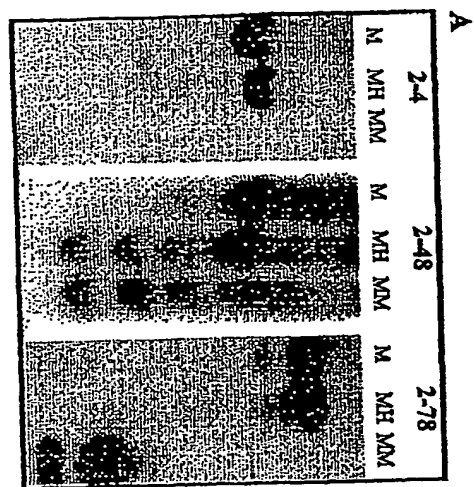


Figure 12

[illegible]

Figure 13

Fig. 14

Figure Sequences not available in public databases

1-5

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1-12

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1-13

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1-20

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1-22

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1-32

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2-6

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2-22

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TGCTGCCCCGCTCTGACCCCCGAGCCCGCGGCCG

2-42

CGGCCGGGGGGCCCCCTGGGGAGCTAGGCCGGGCTCGGGCACAGGCACCGG
CACGGGCACTGGCACC GG CACCGGCACGGGCAAGGGCACCGACCCGACGG
CGGTGGGCGCGGGCCGGGAGCCGCTGCCGCTCTCGGTCAGCACCGTCCGC
TTGAGCGGCCAGGCGCCTCGAGGCGCAGTGGCCCCGGCGGGCGGGCG
GTCCCCGGGGGGCTTGCGCGCGCGGTGCGAGGGCCGGCGGCGCAGCTCGG
ACGTGAGCTCGTGCTTGAGGAAGCGGAACACCTCCTTGCTGGGCCGCGG
CGCTCGGGCTCCAGGGCCAGTAAGCGCTGGAACATGCGCAGCGCGGGCTC
GGTGAAGCGGCGCCACTGCGAAGGCAGCCCCGGCAGGCGGCCCGCTGCC
AGCGCACGAACTCCTCGAAGAAGGCGTCGGCGCCCGACGCCGCCTCCCAC
GGAAGTTGCCGGTGAGCACGCAGAAGATGAGCACGCCGAAGGCCACACG
TCCACGCCCCGTGTCCACCGCCAGCCCGTCGGCGCGGCCCGCCTGGCACAC
CTCAGGCGCCGTGTAAGGGATGGTGCCGCTCACGCGCTTGACGCGGCAGC
CCACGCGGCGCGTCATGCCGAAGTCGGCCAGCTTTACGCGGCGGCACTCG
CGGTGGAACAGCAGCACGTTCTCGGGCTTGATGTCGCGGTGCACCAGCTG
CCGCCCCGTGCATGAAGTCCAGCGCCAGGCCAGCTGCTGCACACAGCGCT
TCACCGTGTCCCTCAGGGAGCCCCACCTGCGGGCGGCCG

2-48 (BpH)

TAAACCAATTTACAGGCAAGTTTCCCTTGAAAACAACTCCTTGCCATA
ATCATCACATTGAGTGACCATCTACCAAATGCTTTACTCCCATGAT
TTCATGTAATATTGACATTCACCCTACAAAGTAGATGGTATTACAGTGTC
TGTTTTACAAGTGAGAAATCCGAGGAACAGGAAGTCAATTTGCCAAGTGT

TGCACAGCTAAATCGAGATTCCAGAGAATGTCACCTCAAAGCTTCTAGTG
GGGCTGTCATGTAGGTTGTGGTCGCTTTGGATAACAGGAGACGCTAAGGA
AAATCAGTACTGGTTACTGAGGATGGAAGAGGCGCARATATTTCAACCACA
GGCGACGAAAACCCCACTTTTAGGCTGGCCACACAGGAGCCCCGAGGAAA
CTATGCGTCCCCCTTCCTCCCCGCCCCCACTGCCCTGGCCTGGCGGAGC
AGCGGCCGCAAGTGTAAGTGYTGTTGCCAGATCGAACCAAGCCCCGGTCC
CAGTGACGAGCAGCGGCCTGCGGGGCCAGAGCGTCTGGGAGCCTTTCATG
ACCCCAAAGCCCAGGGAGGTCCCCGCACCATCGGGCCCCGCGCCCTAGCT
CGGTCCGCCGTCGAGGGTGCTGAAGTCCCCTGCGGGCGCCGGGGAGAAA
GCCCCGGGCTTAGCCTCCTCCATCCCAGCCATCTGTCACCGCCTCCTAG
GCCCCGGCTGGAGCCCCATGGGCGCCTCCCGCGCCTACCAAGGAGCCAGG
GAGACAAGGATCCCGGAGACCTCTGGGGCGCCCTCCAGCTGAGGATTCCG
CCGCGGCTCCCGCAGCCGCTTCTCCCCATTGCGGTGCAGCCACCTGGCCC
AGCTCTCGGCCGGTCTCCCTCGGAGGTCCGAAAAGGGAGAGGGCGGGCCA
GGGCTCCCCGCTGGCCGGAGCCGCAGCCCCCTTTCCCCCTCCCCACCCAG
GGACCTTCCCGGACCCTCCGGGCGCAGCCCTCACCTGCTGCCCCGACC
GCCTCCGAGGAAGGCCCTCGGGCTCCACCTGGCCTCATCACCGCTTCCCT
TATCCGGGAGGAGGAGGAAACTCAACCCTCTAGGCCAGGCCCTGTGCTCA
CTTTAGATACTTTATTTTCGTTTAATTCTTAGGGTTTTAACCCTGAGTTT
AAGGCGAAGGATCCGAGGTTCCGAAAAGCCATGCAGWGCAGGGAGGATTC
AAACAGCCAAACCTGCTGGTCTCCGTGCTCTTGGKAGCGNAAAGAGATT
TWGRKGGAGWAAGTCGTTTTWTAGYTATACTCYCTCTGTGWAAACATAAT
WAAAASTGSMCCACCMCTTSTGGAAAGAARGGCATGSTGSACARCACAR
GGKCTTTTATGAARGGSWCTAARGGAAGATAGCATACCCCCAGCCCTCGT

CTAAGCTTGGTAATTCTCATTTCGCTTTGAACGTTAACMAGAAATTCCAG
GCTCAGTAACCAGTTTCAGGAAAATGCTGTGCGTGAATACAAGAGGAGGC
GCCTTGGCATAGGGRAAGCATTCTGTCCTCTTGATGGACAAGATCCTCCA
CTCCCGTCTTGGCCTGTGACACAAACACCTTGAGTTGTAAATTCCTCGAG
CAAAGTGAGGCATTTTGGCATTTCAGGGGTGGTGAAGTACACACAGGG
AGCCTCAGTACTGTTTATTTGGTGTCTCCATACCTAGCAGACCACATTTT
CCAGGCCCCAAGTAGGGGGTGGGAGGGATCTCATTTTAAAGAGATCAGTT
GATATTTCTCTTGGCAAATCTAGCACAGGACTTTTGTCCCCAAAGACTTC
TGTTCTCACTGCTTGCCACATGCCTGGGCAGCCTAATGCTCTCTACGCC
CATCTTTCYTTCAGGAATGAGTTCCCATCTCTTCTCAACAGTGGACACC
ACTCCAGTGTTCCTCCCACCACCTTTAACTGAAAAACAACTGCCTTTA
CAGAACACTGTGCACACAGGTCAGCCTGGCTCCTGGAAATGCAAACTGGA
GTTTCAAAAGATGAAAACATTCCTGRAGAATTTTGGTGTTTTGGAGAG
TGCCTCTGGGCAGATCACACACTTGTGACAAGTTCCTCAATTGTGAAAAT
TCAATCATGCTTTCCACAAAGAACTGACTTTTCACACTTAACACTGGAGG
TTGCTCATTFTCCCCCAAATCTTGAAGTGGATTTGGGATTAAGATACCAA
AGCAAATGCATAGTTCTTTGAGCACTGCTCCTATCTCATGGTGTCTGCAT
ACTGGCAGACAGACACAGGCAGGAAGTAGGGGGCCTCTGCTGATGGTTTC
CTTGGAGTTAGAAAGGTTTGACACATCCAGCCCAGAGAAGGCAGAGGCTC
CTGTAACCCCAACCCTGCTGCCAGCTGTGAGTGAAGAAAAACAGCTGGAG
GAGGGGGGAGATCTCNACACTCCAGTCTCCCTTAATTTGGMAGGGCTTTT
CTGCTAGCAAACTGTATTCTTTCCTTYTTAAATTTATTGGTAATCACAAA
TTCTCATTATTAGGGACATGGGACATTGGGAGAGGAGGAAMCMCTTTATA
TTWAAAAATTTCCGCTTGGTTCCAAGATGGCCGAWTAGGAACAGCTCCAG

TCTGCAGCTCCCAGCGTGATCGACACAGAAGACGGGTGATTTCGCATTT
CCAACCTGAGGTACCTGGTTCATCTCATTGGGACTGGTTGGACAGTGGGTG
CAGCCCACAGAGGGTGAGCCAAAGCAGGGCAGGGCATCACCTCACCCAGG
AAGCCCAAGGGGTGAGGGGATTTCCCTTTCCTAGCCAAGGGCAGCCATGA
CAGACTGTGCCTGGAAAAATGGGACACTCCCGCCTAAATACTACACTTTG
CCAATGGTCTTAGCAAATGGCACACCAGGAGATTATATCCCGCGCCTGGC
TTGGCGGGTCCCACGCCCACGGAGCCTTGCTCACTGCTAGCGCAGCAGTC
TGAGATCGACCTGTGAGGCAGCAGCCTGGCAGCGGCAGGGGCGTCCGCCA
TTGCTAAGGCTTGAGTAGGTAAACAAAGCAGCTGGGGAAGCTT

2-52 —

CGGCCGCTCCAGGCCCGGCTCCTGCCCCCTCGGCCTCCTCTCCAGGCCAG
AACTGGTTCCCGTCGGCCTCTCCAGGCCAGCTCTCCCGGCCACCTCCAC
GGGCCAGCTCCTGCCTCACGACAACCACGTTCCGGCCAGCTCCTGCCCA
GCTCCTGGCAGCCGTTGTAGGCCCCAGGCTTCCCTGCGTTCAGGCCTCCC
GGACCCACCTTCGGCTTTCCGGCGGCCCTGAGAGACCCGGCTCCTGCCTG
CCAGCGGCCTCTCCCGGCCAGCTGCGGCCTCACGTCGGCCTCCCCAGGC
CACGTTTCCGCCTGCCTCACGGCAGCCCCGGCAGGCCCGGCTCCCGCCTG
CCGGGGGCCTCTTGAGGAGGCTCATCTCGTGCCCCGGCCG

2-59

CGGCCGCGACCCCGCCATCTCTGAGCCACGCCCCCTAGCCAGGGCCGCCC
ACCCACTATCACTGAGGCCCACACCTGCTGAGACCCACACCTGCCGAGGC
CCACACCTGCCCAGGCCCCACCCATTATCACCGAGGCCACACCTGCCGAG
GCCCCACACCTGGGGGATGGGCAGTCGGGGGAGGACGAGTGGTGCCGAGGG
TCTGGGGGGCCCCCTGAACCACCAGGGCGAGGTTCCCGGCTGGGGAGACGC

AGAGCCAGGGCTCTGCACAGGGGGTGCCCTGGGGAGCAGGCATGAGAGCC
ACTTCTGCGAGGTGAGGTCACGAGACAGACGTCAACAAGGGCTGGCCAGA
GAGAAGAGCCGGTCACCCAGGGCCTCGGAGGGAAGGAAGGCTCAGGGACC
CGCGGGACGAAGGCTTGAGAGAAGCCCCTGGGGAGCAGCTTGAGCACAGCG
AGCTCTGGGACAATGGCCAGTGTCCAGCGACAGGGTGTTTCAGAGACGGGG
TGTCCAGCGACAGGATGGGTCCCGGGGGACAAGCGGCGG

2-71

CGGCCGAAGATCGTGACCGACACGCGCACCTTGGATTTGTCGTAGTTGAC
TTCCTCGACCGAGCCGTTGAAGTCGGTGAAGGGGCCTTCCTTGACGCGCA
CGACCTCGCCGACCGTCCACTCGACCTTGGGCGGGGGCTTCTCGACGCCC
TCCTGCATCTGGTTGACGATCTTCATGACCTCCGCCTCCGAGATCGGGGC
CGGGCGGTTCTTGGCGCCGCCGACAAAGCCCGTCACCTTGGAGGTGTGCT
TCACCAGATGCCAGGACTCGTCGTCCATGAACATCTCGACCAGCACGTAG
CCGGGGAAGAAGCGGCGCTCGGTAACGGCCTTCTTGCCGTTCTTCAGCTC
GACGACCTCTTCGGTAGGCACCAGGATGCGGCGG

2-75

CGGCCGCCAGCCCGCCAGAAGCCACAGACAAGACATAGGTAGCCGTAGT
TGGACTGACGGGCAGGGCCGGCGGGGCAGCCCCCTCCGCGTCCCCGGCCG

3-2

CCCCACACCCTCCTCAGCATTGCGGTCTGTGTCCACGCGACTGCCCCAC
GCCCTCCTTAGCATTGCCATCCATGCCCATGTGGCCGCCCCACGCCCTC
CTCAGCATTGCCCCTCTGTGTCCCTGCGGCTAGCCAATGCCCTCCTCAGC
ATTGCCCCTCTGTGTCCACGTGGCCGCCCCACACCCTCCTCAGCATTGTC
CCTCTGTGTCCATGCAGCCGGGCCACGCCCTCCTCAGCATTGCCCCTCTG

TGTCCACGCAGCCGGCCACGCCCTCCTCAGCATTGCCCCTCTGTGTCCA
TGCAGCCGGCCACGCCCTCCTCAGCATTGCCCCTCTGTGTCCACGCAGC
CGGCCACGCCCTCCTCAGCATTGCCCCTCTGTGTCCACGCAGCCGGCCA
CGCCCTCCTCAGCATTGCCCCTCTGTGTCCACATGGTCGCCCCACGCCCT
CCTCAGCATTGCTGTCTGTGTCCACGTGGCCGCCAAGCCCTCCTCAGCA
TTTGCCCTGTGTCCACGCAGCCGGCCACGCCCTCCTCAGCATTGCCCCTCT
ATGTCACGTGGCCGCCACGCCCTCTCAGAATTGCTGCTGNGACACGTG
GCACCCCATGCCCTCTTAAGATTGTCATNCATGCCACGTGGCACCCAC
GCCCTTCTTAAGATTG

3-4

CAGCTGCTCAGCCGAGGCCGATGCTTCCCACTTCCCCATGCCCAGGATG
CCACGTACCTGCAGGTGCGCCACGTACCTGCAGGTGCGCATGTCACCCG
CACGCCACCACATCACCCACAGGTGCTCACGTACCCGCATGCCGCCACG
TCACCCACAGGTGCGCCACGTACCCACACGTGCGCCACGTACCCGCACGC
CTGGCTGTGGAGGGGGAGTGAAGCCTGTGCTTCCCTGCCCATGCCCTCAAC
GCCAAGCAGGTCCCTCCCTCTTCTCTCCTAACTCCTTCCCACTGGCCAGA
AGGCACAATGTCACTTTTAGCTCTGAGCTTCAGATCTGGGTGGAGGGTGG
CAGAACAGCAAGACCCTGGGTTTGGTCCTGGCCACCAAGAGCTGCCTCG
CCACTCGCCGGACCACACACTGGGGCTGTTTCATGGAAAGCCGCATCTCCC
ACTGTCCAAGCCCACATGCTGAGCCGTGCAACATGGAACGCAGGTGTCAA
CCTGGGAGTGGCCTGCACTCAGAAACGGAGCAGGCGTGGGGGAAATCATG
GGCGGAATTGGGAAGGAAGGAAGCGCTGAGGAGTGCTGGGCGTGAGCCGT

GCCACATCAGGGCTGGCGGGGAAGGCACAGAAGGCACAGCCAGAGGGGT
GGGAAATCTGGGAAGGGGCAGGACACGAAAGCCAGGAGAAGGTTCCCTG
GGACGGAGAGCTCCACAGAGCCACGGCCG

3-12

GCGGCCGGGGACCCACGCCATGGTGCCGGGCTATGGGTGTGGGGTCAGCC
AGGGACCCACAACATCGCACTGGCCTGTGGGGTCGGCCG

3-20

CGGCCCGCGTTATATGACATTCCACGTTATGTGACATTCCGGTGTGCCGG
CGTGTGGCCGCGTTATATGACATTCCACGTTATGTGACATTCCGGTGTGC
TGGCGTGC GGCCG

3-30

CGGCCGTTCTCTGTTACCTCTCTCTGGAGACCCCGGCTTCTCCCTGAAG
GCCTGGGAGCCTCACCCACGGCCTGGCCCGGAGAGCGGTCTGATGAGGA
TCAAAAGAAGCAAGGCTGTGGCTGGGACAGGGCACTGCTCGGAGGCCCGC
CCTGGAGGCAGGCGGCCACCAGCCTTCTCTCTCCTTCCCGCACTTTCTCC
GGGCCCCGGTCGCAGGGACCAGCGGGCAGCCTTGGCTCTGGGGCGCCCTC
CTTTCTCCCTGCAGCCCCAGGCGGGCTTCCGGGGGCTGCGCTTCTCTCCC
AGCCAAGGACAGCGCTCACCCGCGCCCCAGTCCCCACGCACCAGCTGTGC
AGCCGCGCGCGCCTCTCTCGTCTCCGTCCAGTGAGTTCTCCGCACTGCAG
AGGGCGAGATCCCGAAGGCCTGGATCCGCGCAGAAGCAGGGAGCACCTTC
CATGGCCGCGCCATCCTCAGCACCGTCCCGCGGCTGCCGCCATCCTCAG
CACCGGAAGGAAAACCAGGCCGCGCCATCCTCAGCACCGGAAGGAAAAC
CAGGCCGCGCGCCATCCTCAGCACCGGAAGGAAAACCGGGCCGCAGCACGG

CCTTGTTGGGCTCCCTCCGAGCTCTCTGCCGCCTTCATGATCCAGCCCCG
GTCTGACCCCCGCTCCTTTCTGGCCTTTGTTCCACCCCCTGTCTGAGCC
TTCCCCAGTCCGGACTCGAGGCCGCTCTGTGCAATGCCACCCTTCGCTAC
CCCGCCTGGTCCAGCGGATCCGCCCCCAGCCTCTCCAGGCCGGCGCCTCC
TCTACCGGGACTCAGCTGCGCGCTCCTCAACGGGCCTCCCCGGCGGCGTC
TGCGCTGCTGGAGTCGGCGTCCGGCTCCTCCCGAGCACCGGGGCTCCTGC
GGGCTCCGCGGCCG

1-102

CGGCCGACNAGGTGTGCGGCACGGGGCCNCGCCAGACTGCAAATGTCATT
ATCTGTTATTTACCACAACAGAGGACGAGAGGCTGCACAAAATTACCGCA
CTTGGCAACGGCCG

1-103

CGGCCGGCCCTGCCCACTGGCTCTGCCGTCCCTAGGCAGTGAGGGGCTTA
GCACCTGAGCCAGCAGCTGCGGAGGGTGCTTTGGGTCCCACAGCAGTACC
GACCCAAAGGCGCTGCGCTCGATTTCTCCAGGCGCCTTAGCTGCTACCCC
AGGGACTAGGGCTCGGGACCCGCACCCCGCCATGCCTGCGTCCCAGCCCA
CCCCTTGCCGTGGGCTCCTGTGCGGCCG

1-cl

CGGCCGCCANNGGGCCGNCCATGCCGGCCCCGGTGAGCGCGGCATCGCCC
TGCTGGAGTTCGCGGGCGGNACAAGCTTTNGTTCCNGAGCACCAGGCCGC
GNTTCGTCGGGNACCTTGNGCGCNTTANNTGGTTAGGGGCTTNNCNGAG
GNGGCCCNNGGTNCCAGNCNGTNNTTTCATCTCTGNTNNGGTNANCCGGCT
CTNTCCTTGGGACGGGNCGN

1-e2

CGGCCGNTGTGGCCACCACGCTCAATGGGAACTCTGTGTTTCGGAGGCGCG
GGGGCCGNCTCGGCTCCCACCGGGACGCCCTCGGGACAGCCGCTGGCGGT
GGCCCCAAGCCTNGGCTCGTCNCCACTGGTCCCGGCGCCCAACGTGATCC
TGCATCGCACACCCACGCCCATTCAGCCCAAGCCCGCGGGGGTGCTGCCC
GCCCAANCTCTACCAGCTGACGCCCAAGCCGTTTGCGCCCGCGGGCGCCA
CGCTCACCATCCAGGGCGAGCCGGGGGCGCTCCCGCAAGCANCCCAAGGC
CCCGCANAACTGACGTTTCATGGCGGCGGGGAAGGCGGNCCAAGAACGTG
GTGCTGTGCGGGGCTTCCCCGCNCCTGCGCTGCAAAGCGAACNTNTTCAAN
CAGCCACCGGGCACCANACCGGAGCGGCCCCGCGCAAGCCCCCGCGGG
GCCCTTGAANANAACCCATGATCNTTCCACCTTTCTTGAACCCAAGGNAA
GCAGNATTTGTCATTCCCCCGCCCAANNAACATNCCTGTCCGGGCCAAAA

CNCAATTTTNCCTACTGNTCTTGGGCACCCCCNCGCGGNTGCAGCTTTCCT
GCAGNATTCTTTTAANCNCTTNCCCCGGGNCAACNNTGGGGCCGGGNAANA
ACCTNCGCGGGCNGCTTTTTTAAAAANTAAGTNGGATTCCCCCGGGGCCTG
GTAAGGAAATNNTNAAATTNNANAGNCTTTATTN

1-g6

CGGCCGCCATCTCGCCGTCGTCCCGCGGGGTGCCCGGGGCGTTGCTCAGG
CCGGCCACGGCGCCGGGGGAGCTCTTCGGCAACCCGTCCATGTCGCCCCGA
GCCCAGGGATCCGCTTACGTGGTGAGGCTCCATCGCGCTCATGGCGGCCA
TGGGGCCCTCCGGGCCAGGGCCGAGCGGGAAATTAGCCCTGCCGGCACCC
GGCCCGATGGGGTTCATGATAGTGTACATGTTTTCACTGGAGTTGGTAGA
ATCTCCAGGGCTAGGCATGATGAGGTGTTCCAGGGGGCCACCTCCTCCT
GGGGGTCCCGTGTAGCTGCCAGGGGATGAGGAGGAGTAGGGGATCGAGTT
TCCACTGGGGCTGGCCACGGGCCACGAATCCTGGGCCCATGTTTCATGG
CAGGCAGGCCTGGGCCGGCAAGGGAGTTGGGTGGGGGTGCGATGCCGCTC
ATAGCTNTGGGGCCCCACGCTGGCATGCCGCGAGGAGGCGTCACCCTNCG
CATTGGGCCGCCATGCTTCGGATGCCCCCTGGGTTCGTGGGGAGGGCTCC
ATGGCGCCAGGGAGGAAGGGATGGGAACCCGGGAGGCCTGCNNGGAGCTGA
CTTAACATNCGCAGGGNNGGNCCGGGACCCCCTGGGAAGCGCCGTNACAT
TAAAGGCTNNCCCGTGAAGGCCCATNACGGGGCATTG

2-109

CGGCCGGAGCATGGGCTTTGCTAATGGTTGGTTCGAGGGTTGTGCCCGCCT
CCACCTCAGTAGGCAACTCTGATAAGACACAGAATTGAAGACTCGCGGGC
GGGCTGGGGCCTGCGCAGGCTTCTCCTTCCCAGAGAGATGAACGCGAACG
TCCACAGAAATAAATGGATGGACGCGGCGTTGAAGCTGGAGTATACACAA

TGCCCCGCTGTGGAAGCAGCTCACAGTTGTCTCCAGCGATGGGTGGGTGC
ATGGACCACTGTGGCCGCGGTACAGTGGAGTGGGGTTCAGCCCGAGAGAG
GAGCGACACGCTGGCACGCCGCAGCACCATGCTGTGTGGAAGAGTCAGAC
ACGCAGGAACAGATGCTGTCCCTCTCCTGTGCAGAGCACACGGCAGCAAG
TCCAGAGGGACGGAAAGGAGTTCAGAGGCTGCAGGGCGCGGCGGGGAAT
GCGCGGTGACTGCCGTGGGCGCGGAAGGGGAATGCGCGGTGACTGCCGT
GGGCGCGGAGGGCGGAATGCGCGGTGACTGCCGTGGGCGCGGANGGGGGA
ATGCGGAATGCNCCGTGACTGCCCTGGGCCCCGGANGGGGAATGCCCGG
TGACTGCCGTGGGCNCGGANGGGGAATGCCCGGTGACTGCCCTTGGGCC
NCGGAGGGCGGAATGCCCGGTGACT

2-a2

CGGCCGCCCCGCTCCGGAACACGGCGGCAGCTCATCTGAATTCAAATTACC
CCGGGAGCCGCGCGATGCCAGCCATAACTCAGCCTGCGGAGGAGTGCGGC
CG

2-c5

CGGCCGATGTCGGCATCGCGATCGGCACCGGCACCGACGTGGCCGTGGAA
GCCGCCGACGTGGTGCTGATGTCCGGCAGCCTGCAGGGCGTGCCGAATGC
GATTGCGCTGTCCAAGGCCACCATGGGCAACATCCGGCAGAACCTGTTCT
GGGCCTTTGCCTACAACACGGCGCTGATCCCCGTGGCCGCCGGCGCGCTC
TATCCCGCGTATGGTGTCTGCTGTCGCCGATTTTGGCGGCCG

2-d10

CGGCCGGGCTNTTTGATTGGCTGCCGCGTCGGCGATCCACGCCACAATTG
TTCCCTAAGACCGTCTGCCGCCAGCGAGCGCCAGGTGCGGAGCGGGCGTT
AGAAGTTGCTGGCAGTCAGAGGCAGGGGAGCTGTCACTCGCGGCGAGCCG

GGCGGCGGCCAGGGCGCAAAGTTGAGAGCAGTCTCTAGTCTGAGCCTTTC
AGTCGCCTTCCAGTATCATCAGTACCACGGGCTCCACCTTGCTGCGGCCCC
CTCAGCAACCCAGTGACCTGCCACTCGACCAGGTÀGGTAGGCCGAGGCA
CCCGGGCGTCGGTCATCGCGCCTTCGCCGCCCTTTGCGGCCG

2-e12

CGGCCGAGGTGGTCGGAGTCGCAGGGCCCGTGGAAGGCCTCGGGGAGGAG
GAGGGTGAGCAGGCGGCAGGCCTGGCCGCAGTCCCCAGGGCGGGAGCGC
CGAGGAGGACTCAGATATCGGGCCCGCGACGGAGGAAGAGGAGGAGGAGG
AAGAGGGGAACGAGGCGGCCAACTTCGACTTGGCGGTGGCCACCCGTCGG
TACCCGGCGGCGGGCATTGGCTTCGTGTTCTGTACCTGGTCCACTCCCT
TCTCCGCCGCCTCTATCACAACGACCACATCCAGATAGCGAACCGTCACC
TCAGCCGCCTGATGGTGGGGCCCCACGCTGCTGTGCCCAACCTCTGGGAC
AACCTTCCCCTGCTGCTGCTGTCCAGAGGCTGGGTGCAGGGGCTGCAGC
CCCAGAAGGCGAGGGCCTCGGCCTGATCCAGGAGGCTTGCGTCGGTCCAG
GAGGCCGCGTCGGTCCCAGAGCCTGCAGTGCCAGCTGACCTGGCCGAGAT
GGCCAGGGAGCCCGCGGAGGAAGGCCGCAAATGAAAAACCCCAAAAGAA
GGCCGCAGAGGAAGAACTCACAGAGGAGGCCACAGANGAACCGGCCCG

2-e3

CGGCCGGCAAGGCTCAGGACCTGCAGGCCATGGAGTGGCGAGGCTGCCAT
GGAGTGGCGAGGCTGCCGTGGAGCGCGGAGGCCGGGTACGCCTGCGCGTG
GAGCGCGAAGGCCGGGTACACCTGCGCGTGGAGCGCGGAGGCCGGGTACA
CCTGCGCGTGGAGCGCGGAGGCCGGGTACACCTGCGCGTGGAGCGCGGAG
GCCGGGTACACCTGCGCGTGGAGCGCGGAGGCCGGGTACACCTGCGCGTG
GAGCGCGGAGGCCGGGTACATCTGCGCGTGGCACGCGGAGGCCGGGTACA

CCTGCGCTCATCGCACACCAGCGCCACGCCAGACGTACTCGCGGGAAG
GACAGCNTTTTNTANCNAAAAANCGAATGGTCAACCCGNTTTANTTAACA
CGGGCCANCCCGGAAACAGCCCGACACGGACCGNGACGGGCGG

3-100

CGGCCGGCCCAGCCCTGCCATGCCCCGCTCCTCAGGGGAGTACGCCCGCG
CATCGGTGCCGGAGAGGGGAGCCAGGCTGGCCTGCCGGCCG

3-110

CGGCCGCATTTTATAGTCAGACACAACCACAACATGGTTGTGACCGGGCA
GTCGAACCCTCAGGATCGACCCAAGAGACATGAAACTACCCACACAAAGG
CTGCTATGGGAACATGCACGACACTCCTCCTTCCTAATAGCCAAAACACG
GCCG

3-e11

CGGCCCGCCTCCAGAGTTTCAATATGGACCTCCGAAGGAGGCACCTCCAC
CTCCATGCCAGTGCTGGTCTCCTGACAAGAGAGGGTTGCGCTACTAACTG
GCATTAGGTGGAACGTGTCGCACAGAGGACACGGCCTTCTGACAAGGTTCA
AAGCTGGACGTGAGAGAGAGAGTGGCAGATACACCCTCACTGACGTGAGC
CCCTGGCAGGCAAACGTTTTCCAAAGGCTCGGCTTGGGGAAGCTCCCTTC
CTATTGGCCTTGGCCCTGAGTCTGAGAGAATGGATGCCCAGTGGCTCAAG
AAGGGGCATACAGAGGCAAGGCCTAGGAGGAGAGCAGCCTGCCCTCCCAT
TTCAGAGCGAGGCCCTGCGTCTTGCCAGCCCTCCTAAGCCCTGGGTGTG
GCGGGATTGAGTGCGAGAGCTGCCAGATGAAACACGTCAGCCCGGCCG

4-b10

CGGCCGTCCCCAGGAGAGAAAGAAGCCAGAGAGCATGTCCAAATGCAGT
GCTGGGCCTGTGTGGGGCTGGGGCGGCTGCGGCCG

4-c6

CGGCCGCGTGTGGGGCCAGGCCCTCACCTCCCTGTGCTGGCAGCACTGA
CCGAGTGCCTGGGCCCCATTCCCTGAGGATGGGCCACCCAGAGACACCTG
GGCTCAGATGTTACAGTGGCTGAGAATGGGAAGAGAGAGGGCAGCTGT
CCTGGGGTGGAGTTTCCGCAGATCACAGCAGGTGGGCAGGGGCCAGGCTC
AGGCTTCTTAGGAACTCGGCCTCTGTCCCCACAGAGGGATCTGTCATCTG
TGTGCTGGGGTTCATCATGTCCTCGGGGTGTGTGTGTCCCTGAAATCCC
TGTCCTCTGTCTCCGTCATGCCCTGCTGGCTGTGTGGTGGCTACCCT
GTCCCTNTGGCCCTGGGTAANCTTGGCAGANNCCGGNTTCTTTNGCTTC
CAAATAAAGGAATANACCCCAAAGGGTCATTCTCTAACATGGTCAGGAGG
AGGGCTCTGGGAGAGGTGTCGCTGTGACTGTGGGCTCATGACANGCATGA

ACCCCTTGNGGGAAGCGGGGGCCCCCTGTGATCCCTTTCTATTCAATTTTC
TTCGTCTTTCCCCACAAAATGCTGTGTGCTGTGGACCCACCTTGGGGNTC
ATGGAGTGGGCCACCGGGGGCCACCCTAAACACTTGTTGCNCCAANGGT
CGNCCCGCCTTCTGNNTTGNGGGTCCCCCGTGCCCCCT

4-c10

CGGCCGTCTCCAGGAAGGACAGCCTGGCAGGCCCCGGGGGTCCCTTGGCT
TGGAGCCCCCAGCCCAAAGTCCCCTCCTTTCTCCCAAGATGGGGTGGCTG
GTAGCCAGGGTGGTGGGTACCTACTGCACACGTAGGGAAACTGAGGCCAG
GGAGGCCACCCAGACCTTGCCCTGGCCCACTGACCTGTAAGCGTCCACC
GTGAACCCGCTGCCCACTGGCCCCCTGTTCCCCACGGGCCTTCCCTGCC
TAGCCCAGGCCCCACCCAGGCCCTGTACCTCAAAGGGCTCCCCGGGG
CCAGCGGGAAGATGCTAGACACCTGCTCCGGGGCCCCAGCGGCCG

4-d5

CGGCCGCCAAGAAGGCCGCGCCCGCGAAGAAGGGCGTCAGCCGCGTCGTT
GGCAGCAAGACACCGGCCACCAAGACCATCAAGGNCGGCGCGGCCAAGCC
GGTGGCGAAGAAGGCGGCTCCGGCCAAGAAGGCTGCTCCGGCCAANAAG
CGGCGCCCGCCAAGAAGGTCGTCGCCACGAAAGCCCCGGCCAAGAAGGCT
GCAGCCAAGAAGGGCTGATGCGTCTCCTTCTAGTCGCCGTGGGCCAGCGC
CAGCCGGCCTGGGCCGACACGGCCTATGAAGACTTCGCCAAGCGCTTTCC
GCCCCGAGCTGAGGCTGGAGCTGAAGGCCGTCAAGGCCGAGACACGCGGCA
GCAAGACGGCCG

4-g6

CGGCCGTCAGCCATCGTAATGACATGTCTGTGGGTTGCCCTGTGCCGCCA
GGCTGGGCTGTCGGAAGCACCCAGCGACGTGTCTGTGGGTCCGCCCCGTG
CCGCCAGGCCGGGCCATCGGAAACACCTGCAGTAACCGGAGTGCCCTCGC
TGATAGCCCTTGTTCCGGGGCCTCGTCCTGGGCTGTGCAGAGCTCCAGCC
CTAGCCCCAGCCCCAGCTGCAGGCGGCCG

Figure 14 (continued)

Clone 2-12 Glioma tumor suppressor candidate gene 19q13.3

TGGA CTCACCGCGGTGGCNGCTGACGCCAGCGTCACGGGCTCCGAGGGGC
CAGCCCGCCCCGAGGCCAGGTAGCCGCTGACGGGCACCTGCTTGGCCAGGA
GCTGGGAGGTGGGCGTGTTGAGCGCCATGACGGGCTGGCCCCACCACTGG
ATGGGCGCCAGGCCAGCGTGGCCGCCGTGGCACCCCCAGGGCTGCCATT
GGGCAGGCCTTGGAGGCCCGGGATGGGCTGCAGTGTACATTGCCAGGC
CCACAGGCTGCAGGAAGGGCTGCACACTCAGGGCCTTGTGACCACGTCC
TGGGGCGGCACCAGGGCCTGGTGGGTCAGCACGGTGGGCGGGCCCTGCA
GCCCCAGCAGGTCGGTGCTGCTGGGAAGAGGGCTTGGGGCCCCGCAGCCA
C

Clone 2-36 Zn-finger protein and novel arginine vasopressin w/ 9 CpG islands Chr. 20

CCGCGGTGGCGGCCGCCCCGTCTGGGAGGTGGGGAGTGCCTCTGCCCAGC
CGCCACACCGTCTGGGAGGTGAGGAGCGCCTCTACCTGGCAGCCCCATCT
GGGA ACTGAGGAGCGCCTCTGCA

Clone 2-37 Relaxin 1 w/ 4 Cpg islands Chr. 9p23-24.3

CGGCCGGGCAGAGGCGCCCACTTCCCAGACGGGGCGGGCCAGGCAGAGGC
GCTCCCCACCTCCCAGATGAAGGGCGGCTGGGCAGAGGTGCTCCCCACCT
CCCAGGCGGGG

Clone 1-1 Chr. 7q21.2-q31.1

CGGCCGAGATGCACTCAGATTTATGTTGTGAATTTGTTATGTTCAAGTAAT
TTGATGGTGTATTCTTATGCAATGAGATCTGGATGTCATTTCTGGTTCTGCT
AATTAGAACATCTGTGACCTTGATCAAGCAAGAACTTTCTCTTGTGGAC
CTCACATCCTACAATTGTATATTGTCCTGCATGTCCCTCAGACACTTTTCGT
TTTTCTTCAGTCTTTTTTCTTTTTGTCCTTTAGATTGGATAATTTCTGATCTT
CTGAGAATTTTTTTATTATCTGCAACTTGCTGGGTTTTTCTTAGAATTTAG
TTATTTTTTTGTATTTTTTA

Clone 1-19 Chr. 11

AGGGGTGCCTCTGCGCCCTAAAGAAACCGGGGGAGCCCCACAACCCCTCC
CCCACCAGGACACTAAAAGGCAAGCTTTCGGTACAGTGAGACATCAAAGC
CTCCTAGGCCCTGAGTCAAAGGTATAGCCGTGTAATATCCCAGTGCCAGC
TCTCCGGCTGCGGGGAGCCTGGCGCAAAGCTTCCAAGCCTTCCTTGTTCAA
AAA

Clone 2-16 Chr. 21q section 981105

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GTCAAGGGGGACCTCTTGCCATCGGCCTCCAGGGGCCGGCCACCTGCAG
TTTTGGGGCCAGCTGGAGGTCAGCAGGGTGGACTCACAACCCCTGAGT
TCAGGTACAGGGAGCTGTGGAGACAGGCCACCCAGGCTGACCTTCCCCA
NAGCCTTGCTGT CACGGAGAGGAGGGGGCGTTGGAGGAANGGCCACAAA
TGCNNGAGAGGGGGCAATGGCCTGNGACAAGATGGAGAACAGCCACCCG
TTCCCCAGTACAGCCAGGT CANGACACGGATCCCANCAAGCCCTTTGGAT
GGGGAGACTGAGGTACAGCTGATGACTCACCTATGTGATAACAGCTGTG
AGAGCCGGAGTGGGGATGCANACACGGAGGTGGCCAGTGGNCACCTNCN
AAGACTCAACATCCANGGCGATGACGCCAAACAGTCAAGGCGTNAGAAC
CCCCNANANNAAGAGTGAGTGNCATTACCTAATA

Clone 2-2 Chr. 1qtel 1920-c104t3

CGGCCGGGCGGAGGGGCTCCTCACTTCTCAGACAGGGCATTTCGGTCAGAG
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CCAGACGATGGG

Clone 1-12 Chr. 16 clone LA16-305F3 (2CpG islands)

CGGCCGCGGACCCCCGACCTCGACCCAAACTGCATGCGGCTGAGGACCCC
CAAGCCAGGCAGACGCCAATCCAGACCCACGNNNNNNNNNAAGANCG
GTTTTTTTGCCCTTTTGACGTTTGGGAGTCCACGTTCTTTTAATAGTGGGA
CCTCTTTGGTTNCAAAAANNGGNAANAT

Clone 1-7 Chr. 16 clone LA16-361A3 (2 CpG islands)

CGGCCGGTGCCAAAGGTCCTGTGTGCCCAGAAGAAGTGAATGGTTTNGGC
CAGGTCAGGCAGAAGGACCTGGTTGTGGCAGCGCTGACAAGAGAGCACC
CCAGATCCATCCCTTACAAAATGATCGAGGGGCTTCTTCCAGAGGGCACC
GTCTGGTTCCCTGAGGGGAGTGCAGCAGCCCTGACATAGCCTTCAGGAGC
CGTGGCAGAGCTGCAGAGGGGACCCAGCAGTGGGGCCCTGACAAGGAC
GAGGTGCACCACCATGGGGCTTCCCACTGAACTCTCGGCGCCAGGACGAG
CCAAGGGACGGGGGCGGCGCCCANCCANACTCAAGCTCAGGTCCCTTGG
GTCCCCGCGGGGACACCTTCGACAGCAGGTTCTTGGGGCCACCTTCTGC
CCCACACCATGAGANAAAACATTGCAGGACGAATTNCTNCTTTGCCCCGC
AGCCACGCGCCTNTTTCCAAGGTAGGCCCTNGGCCCTGGCCCCATTGA
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Clone 1-5 KIAA 0614

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CGAATCTTANCGCACAAAGGGGCCTNCAAGCTTCGGGTCTTAATNATTTGA
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Clone 2-43 cDNA FLJ12750

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TCTTCCCCCACCCTGTGGGACCTCATCTTCACGTGCCATGTGTGCTGAAG
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Clone 2-48 RP11-393 M18 Chr.1

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CTTNTCCCANATGGGGCGGCCCCGCTCTTATAACTANTGGATCCCCCGGGC
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GCCGCCGCTTAAACTTGGGCCCGNANATNTTTTTTACCAAACGGTTCTTTG
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AAGANTTGGCCGGCCAAAGCCCNNTGGNAAATTGGGGCGAAAANTGGGGG
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GNCGGGGTTGGTTGGNTGGGTTTTACCNCCGCCAANCCGTNGAACCCGCT

TACCAACCTTTGGNCCAGCGGCCCCCTTAACCGGCCCCCNGNTTCCTTTTC
CGCNTTTTCCTTTNCCCTTTTCCTTTTCTTNGGNCCNCNNTTTCGCCCCGGC
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Clone 2-52 BAC in Chr.14

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GAAAGGAGGAGACCTCCGCCTGGCAACCGCCCCGTCTGAGAAGTGAGG
AGACCTCCGCCCCGGCAGTCGCCCCGTCTGAGAAGTGAGGAGCCCCCTCG
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GCCCCGTCCGGGANGGAGGTGGGGGGTCAAGCCCCCTTACCGGCCNGTCNTTTC
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Clone 2-64 12 BAC RP11-588G21

TGGGCTCCACCGCGGTGGCNGCCGTGGCTCTGTGGAGCTCTCCGTCCCAG
GGAACCTTCTCCTGGCTTTCGTGTCCTGCCCTTCCCAGATTTCACCCCC
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CACGCCCAACACTTCTTAAGCGCTTCCTTCCCTTCCCAATTCCGCCCCATGAT
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CACCCAGATCTGAAGCTCAGAGCTAAAAGTGACATTGTGCCTTCTGGCCA
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GCATGGGCAGGAAGCACAGGCTTCACTCCCCCTCCACAAGCCAGGCGTGC
GGGTGACGTGGCGACCTGTGGGGTGACGTGGGCGACCTGTGGGTGACGTT
GGCGGCATTGCGGGTGAACGTGACNACCTTGTGGGTGATGTGGTGGCNTT
CCGGNTGACATTGGCNACCTTCAAGGTG

Clone 2-65 RP11-402B2

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A
GAGAGAGAGAGAGAGAAACAATAATAAATGTCTCCAAATAAGAAGTCAT
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TCTACAAGACCACTGTCTGCTACCTAAAATTTAGAAGGAATAAAAACTC
TGAACCTAGATTGAGGCTTCCCAAACCAAGAGCCAAACCTCAACTTCAG
AAATTCCTGGCAAACCTATGTATTAGCTAGTACATGATAAAATGAAACCTC
CATCCTTGTTAATTCCTTACGTGCAGAGCTGTTTCATATTAAATAATGTCTCT
TTTGTTTTACTCATGCTTTGTTTTTACTTATACTTACGCATTTCTGAACAA
ACGATAGCAAAGCAAAAAAACAACAAAAAACAACCTTTATTCAG
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Clone 2-70 RP11-349E11 from 7p14-15

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GGCAGAGACACTNCTCACTTNCTATACAGG

SEQUENCE LISTING

<110> Feinberg, Andrew
Strichman-Almashanu, Liora
Jiang, Shan

<120> METHODS FOR ASSAYING GENE IMPRINTING AND
METHYLATED CpG ISLANDS

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<150> 60/206,158

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 ggggcgcctt ctccagcatg gcggccg

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 <211> 110
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gccccacgcg ggggtgcgatc tctgcgggag ccggccg	157

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cgccg	126

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<211> 194

<212> DNA

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cgcaggcgcg gctgccctcg aacaggatcg ccatggcggt gcgcgggtc tccacccgca	180
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 gacatggcac atagaaccgg agatgcactg cccggggccat tgtctctgta ggaaaggcgg 180
 ccg 183

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 gcggccg 67

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 cgcttannt ggtaggggc ttncnngag gnggccnng tncagneng tnntttcac 180
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cgggccaaaa	cncaattttn	ctactgntct	tgggcacccc	cnggcggntg	cagctttcct	600
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ggtgaggtct	catcgcgctc	atggcgggcca	tggggccctc	cgggccaggg	ccgagcggga	180
aattagccct	gcccgcaccc	ggcccgatgg	ggttcatgat	agtgtacatg	ttttcactgg	240
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gggggtcccc	tgtagctgcc	aggggatgag	gaggagtagg	ggatcgagtt	tccactgggg	360
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agggagttgg	gtgggggtcg	catgcccgtc	atagctntgg	ggccccacgc	tggcatgccc	480
cgaggaggcg	tcaccctncc	cattggggccg	ccatgcttcg	gatgcccctt	gggttcgtgg	540
ggagggctcc	atggcgccag	ggaggaagcc	atgggaaccc	gggaggcctg	cnggagctga	600
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<212> DNA

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ggcagtgggg	ccctccgca	tgagagacgc	gggcctcctg	gtggagcagc	cgcccctggc	180
cggggtggcc	gccagcccga	ggttcacctt	gcccgtgccc	ggcttcgagg	gctaccgcga	240
gccgctttgc	ttgagccccg	ctagcagcgg	ctcctctgcc	agcttcattt	ctgacacctt	300
ctccccctac	acctcgccct	gcgtctcgcc	caataacggc	ggggccgacg	acctgtgtcc	360
gcagttttcaa	aacatccctg	ctcattattc	ccccagaacc	tcgccaataa	tgtcacctcg	420
aaccagcctc	gccgaggaca	gctgcctggg	ccgcactcgc	cccgtgcccc	gtccggcctc	480
ccgtcctca	tcgctgggtg	ccaagcggag	gcattegtgc	gccgaggcct	tggttgccct	540
gccgcccggg	gcctcaccoc	agcgctccc	gagcccctcg	ccgcagccct	catctcacgt	600
ggcaccccag	gaccacggct	ccccggctgg	gtacccccct	gtggctggct	ctgccgtgat	660
catggatgcc	ctgaacagcc	tcgccacgga	ctcgcttgt	gggatcccc	ccaagatgtg	720
gaagaccagc	cctgaccctt	cgccggtgtc	tgcgccccca	tccaaggccg	gcctgcctcg	780
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<212> DNA

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<222> (1)...(1578)

<223> n = A,T,C or G

<400> 42

cgggccgctgg gtttgttttc acgtgccgat cggatttttc tggctactac tgcagncgc 60
 tgctctcggg cgccccgaag ccgcaggtac agctcccccc aagactcgtg ctctctgtggc 120
 ttttcttcct tgaagtccctg gaggcaatga atcctccata attcatctgt ctctcgagcg 180
 agtgccgcat tgtctttctc tgtgcggtac ggctgatcgg gcgtccaccc ttccagaacg 240
 ggttcaagaa ccgagtaggg gaccccttcc acgtcgccga gggcgctccg attgttctta 300
 ggcacccgga ggcactgctg gcgcagcgtc ggcacctgga gctggcaggc aggcctggag 360
 cccgagtaca ccggcatctt agcgttcaact ctgcgtccag ggaaagcagc ttctctctgg 420
 agcgttggcg cggagagtgc ttctgggttt gcctgggagg tcatggcctc aaaagcggac 480
 agcagatcgt agttggcctg catccaggcc tctgaggggt cccagagctc cgagaagaca 540
 tggctgggca ccgttttccg cccggcgga tcaagcgccg gccgcctgca gcctctctga 600
 ctggcttttc tggacaggag gcgattttctg agccgaagcc ccgcgggacg atttgtgccc 660
 cttgctgtgg cttccccaccg cttccccctg ggggtggccc tggcagcctt gaccagcag 720
 agggccgccc tgagcggcgt gagcgtggcc tcttccgggt tgctccccgg gcacagcggg 780
 ctcagggccc tcgggcatcg ggaggggagc ggtgcgcgtt ggaggggtccc gatgggggccc 840
 ggaatcagct gggggccattc tggggcgctt tctctcggct ctgggctcgc gactgggaga 900
 cctcgggtga ggtctctgtt gccccggagg tgttctgcgt gctgtccgtc tgtgtcagg 960
 gctgtgagat gggctcctgg gggccgtcgc gttttctggg aagccccagg ccttttcccg 1020
 ctctgaaga gcctccccga agcgtctgcg ggaagcgtc tctcaggggt cctgcggggtc 1080
 agggccggtg tttcgggtcca cgagcaccag cttcttccac cgggcccgtc agtctctggc 1140
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 cgccaggatg tctcggttca tgggcaaggc ggagagtctc tgcaaatatt tctctagctt 1260
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 ggacctgccc gccatctcgc cagagctgtg caggcgtcgc tgtcctcgcg gtcgaggctc 1380
 tgtccagagc cggggcgccg gcacaggcag tctgggggtg ccggctcctc ctgcccgggtc 1440
 gccaggcgcc gacctcgga tgtggagtca cagcctggag cgagctgggt cctcggagca 1500
 gcgggcccact tggctctgga cgcgggtcct tgcagacagc tgagcaggcc cgcttctgtt 1560
 cctcgggatg tgcgcccg 1578

<210> 43

<211> 102

<212> DNA

<213> Homo sapiens

<400> 43

cgggccgccc ctccggaaca cggcggcagc tcactctgaat tcaaattacc ccgggagccg 60
 cgcatgcca gccataactc agcctgcgga ggagtgcggc cg 102

<210> 44

<211> 243

<212> DNA

<213> Homo sapiens

<400> 44

cgggccgatgt cggcatcgcg atcggcaccg gcaccgacgt ggccgtggaa gccgcccagc 60
 tgggtgctgat gtccggcagc ctgcaggcg tgccgaatgc gattgcgctg tccaaggcca 120
 ccatgggcaa catccggcag aacctgttct gggcctttgc ctacaacacg gcgctgatcc 180
 ccgtggccgc cggcgcgctc tatcccgct atgggtgcct gctgtcgccg atttttgagg 240
 ccg 243

<210> 45

<211> 342

<212> DNA

<213> Homo sapiens

<220>
 <221> misc_feature
 <222> (1)...(342)
 <223> n = A,T,C or G

<400> 45
 cggccgggct ntthgattgg ctgccgcgtc ggcgatccac gccacaattg ttcctaaga 60
 ccgtctgccg ccagcgagcg ccaggtgceg agcgggcggt agaagttgct ggcagtcaga 120
 ggcaaggagg ctgtcactcg cggcgagcgg ggcggcgggc agggcgcaaa gttgagagca 180
 gtctctagtc tgagcctttc agtcgccttc cagtatcatc agtaccacgg gctccacctt 240
 gctgcggccc ctcagcaacc cagtgcacct gccactcgac caggtaggta ggcggaggca 300
 cccggggtgc ggtcatcgcg ccttcgcgcg cctttgcggc cg 342

<210> 46
 <211> 443
 <212> DNA
 <213> Homo sapiens

<220>
 <221> misc_feature
 <222> (1)...(443)
 <223> n = A,T,C or G

<400> 46
 cggccgggcaa ggctcaggac ctgcaggcca tggagtggcg aggctgccat ggagtggcga 60
 ggctgccgtg gagcgcgagg gccgggtacg cctgcgcgtg gagcgcgaa ggcgggtaca 120
 cctgcgcgtg gagcgcgagg gccgggtaca cctgcgcgtg gagcgcgagg gccgggtaca 180
 cctgcgcgtg gagcgcgagg gccgggtaca cctgcgcgtg gagcgcgagg gccgggtaca 240
 cctgcgcgtg gagcgcgagg gccgggtaca tctgcgcgtg gcacgcggag gccgggtaca 300
 cctgcgcgtc tcgcacacca gcgccacgc ccagacgtac tcgcgggaag gacagccttt 360
 tntancnaaa aancgaatgg tcaacccgnt ttanttaaca cgggccancc cggaacacgc 420
 ccgacacgga ccngacggg ccg 443

<210> 47
 <211> 383
 <212> DNA
 <213> Homo sapiens

<220>
 <221> misc_feature
 <222> (1)...(383)
 <223> n = A,T,C or G

<400> 47
 cggccgcaag gagagcctcg atggcttcgt ggagaccttc aagaaagagt tgtccagaga 60
 cgcttatcca ggaatctacg ccttggactg tgagatgtgc tacaccacgc atggcctana 120
 gctgaccgcg gtcaccgtgg tggacgcgga catgcgagt gtgtacgaca ccttcgtcaa 180
 gcccgacaac gagatcgtgg actacaacac caggttttcc ggagtcaccg aggcgcagct 240
 cgccaagacg agcatcacgt tgccccaagt ccaagccatc ctgctgagct ttttcagcgc 300
 ccaaaccatc ctcatcgggc acagcctgga gagcgacctg ctggccctga agctcatcca 360
 cagcaccgtg gtggacacgg ccg 383

<210> 48
 <211> 598
 <212> DNA
 <213> Homo sapiens

<220>
 <221> misc_feature
 <222> (1)...(598)
 <223> n = A,T,C or G

<400> 48
 cggccgagggt ggtcggagtc gcaggggccc tggaggccct cggggaggag gagggtgagc 60
 aggcggcagg cctggccgca gtccccagg gcgggagcgc cgaggaggac tcagatatcg 120
 ggcccgcgac ggaggaagag gaggaggagg aagaggggaa cgaggcggcc aacttcgact 180
 tggcggtggc caccgcgtcg taccggcgcg cgggcattgg cttcgtgttc ctgtacctgg 240
 tccactccct tctcgcgcgc ctctatcaca acgaccacat ccagatagcg aaccgtcacc 300
 tcagccgcct gatggtgggg cccacgcgtg ctgtgcccaa cctctgggac aaccctcccc 360

tgctgctgct	gtcccagagg	ctgggtgcag	gggctgcagc	cccagaaggc	gagggcctcg	420
gcctgatcca	ggaggcttgc	gtcgggtccag	gaggccgcgt	cggtcccaga	gcctgcagtgc	480
ccagctgacc	tggccgagat	ggccagggag	cccgcggagg	aaggccgcaa	atgaaaaacc	540
cccaaaagaa	ggccgcagag	gaagaactca	cagaggaggc	cacagangaa	ccggcccc	598

<210> 49
 <211> 677
 <212> DNA
 <213> Homo sapiens

<220>
 <221> misc_feature
 <222> (1)...(677)
 <223> n = A,T,C or G

<400> 49	
cgggccgacgg	60
aagtaaggca	120
gtgtccagcc	180
ggaccacccc	240
tttggtcaga	300
gggtccccga	360
cgtccagtgg	420
cgtttgcaaa	480
ggtcgtggga	540
ggctgcgggg	600
ttttggaant	660
accaagctta	677

<210> 50
 <211> 669
 <212> DNA
 <213> Homo sapiens

<220>
 <221> misc_feature
 <222> (1)...(669)
 <223> n = A,T,C or G

<400> 50	
ccccacaccc	60
gcattttgcca	120
tccttgccgc	180
acaccctcct	240
ttgcctcttg	300
tgccagccgc	360
cctcctcagc	420
cctctgtgtc	480
gccgccaaag	540
atttgccctc	600
ggcaacccat	660
aagatttgc	669

<210> 51
 <211> 91
 <212> DNA
 <213> Homo sapiens

<400> 51	
cgggccggccc	60
ggagaggggga	91

<210> 52
 <211> 154
 <212> DNA
 <213> Homo sapiens

<400> 52	
cgggccgcatt	60

caggatcgac ccaagagaca tgaaactacc cacacaaagg ctgctatggg aacatgcacg 120
 acactcctcc ttctaatag ccaaaacacg gccg 154

<210> 53
 <211> 89
 <212> DNA
 <213> Homo sapiens

<400> 53
 gcggccgggg acccacgcca tgggtgccgg ctatgggtgt ggggtcagcc agggacccac 60
 aacatcgac tggcctgtgg ggtcggccg 89

<210> 54
 <211> 113
 <212> DNA
 <213> Homo sapiens

<400> 54
 cgccccgcgt tatatgacat tccacgttat gtgacattcc ggtgtgccgg cgtgtggccg 60
 cgttatatga cattccacgt tatgtgacat tccggtgtgc tggcgtgcgg ccg 113

<210> 55
 <211> 914
 <212> DNA
 <213> Homo sapiens

<400> 55
 cgcccggttct ctgttacctc tctctggaga ccccggttcc tccccgaag gcctggggagc 60
 ctaccccacg gcctggcccg gagagcggtc gtgatgagga tcaaaagaag caaggctgtg 120
 gctgggacag ggcactgctc ggagggccgc cctggaggca ggccggccacc agccttctct 180
 ctcttccccg cactttctcc gggccccggt cgcagggacc agcgggcagc cttggctctg 240
 gggcgccctc ctttctccct gcagccccag gcgggcttcc gggggctgcg cttcctcccc 300
 agccaaggac agcgtcacc cgcgccccag tccccacgca ccagctgtgc agccgcccgc 360
 gcctctctcg tctccgtcca gtgagttctc cgcactgcag agggcgagat cccgaaggcc 420
 tggatccgcg cagaagcagg gagcaccttc catggccgcc gccatcctca gcaccgtccc 480
 gcggctgccg ccatacctcag caccggaagg aaaaccaggc cgcggccatc ctacgaccg 540
 gaaggaaaac caggccgccc ccatacctcag caccggaagg aaaaccgggc cgcagcacgg 600
 ccttggtggg ctccctccga gctctctgcc gccttcatga tccagccccg gtctgacccc 660
 cgctctcttt ctggcctttg ttccaccccc tgtctgagcc ttccccagtc cggactcgag 720
 gccgctctgt gcaatgccac ccttgcctac cccgcctggt ccagcggatc cggccccagc 780
 ctctccaggc cggcgccctc tctaccggga ctcagctggt cgctcctcaa cgggctctcc 840
 cggcgggcgtc tgcgctgtgc gagtcggcgt ccggctcctc ccgagcaccg gggctcctgc 900
 gggctccgcg gccg 914

<210> 56
 <211> 641
 <212> DNA
 <213> Homo sapiens

<220>
 <221> misc_feature
 <222> (1)...(641)
 <223> n = A,T,C or G

<400> 56
 cgcccgggcgc ttcccgccacc tcccggcgct gctgctacac cggcgccgcc agcatctgcc 60
 agagcggccc cgcgcgtgcc cgctgtgcgc ccgcaccttc cggcagagcg cgctgctctt 120
 ccaccaggcg cgggcgcacc ccttggggac aacctctgac cctgctgccc caccaccacg 180
 ctgcgcgcag tgcgcgcgag ccttccgaag cggcgccggg ctgcggagtc acgcgcgcac 240
 ccacgtgtcc cggagcccca cgcgaccccc tgtctcagac gccaccagat gtggcgtgtg 300
 cggcaagtgc tttggcaaga gctctacgct gacgcgacac ctgcaacgca ctggggggan 360
 aaaccttnna gnngcccgan tgnngnaagg gcttctggag agccccagct ggtgcggcac 420
 cagcgcacac acacnggcga aaagccgtac gcatgtggcg actgtggacg ctgttnagcg 480
 agagttccac gcttnttgcg ccacggcgcg anccatnaag ggcgagcggc cacatgcgtg 540
 cgccacttgc ggnaagggtt tcgggcagcg ctccacctgg tgggtgcacca gcgcattcac 600
 acnngcggaag aagcctttgc gtgccccgna gtggcggggcg g 641

<210> 57
 <211> 428

<212> DNA
<213> Homo sapiens

<220>
<221> misc_feature
<222> (1)...(428)
<223> n = A,T,C or G

<400> 57
 cgccgcgcgc gttccggctc ccgagccccc cctgcgcgcg gcctcctcgg cgcagccatc 60
 ctcttggctg ccgcggggcg caaagccccc ggcatctgcc atttgtcatt cagcccgctc 120
 gtaccgcccc gagccttgat ttagacacgg ctggggcgctg ctctggcctc actctccggg 180
 cgggtgcttg acggacggac ggacggggga gccgtgctca cagctcanca gcgcggggcc 240
 ttggcgcgcg gggcgcttcc ccgggtcgcc gtcattggcc cggaggtgga cgcgcgagcg 300
 gnetcgctg agctccgggg gtcgtcgccc cgcaaggtag nttttgggtg ctcccgttc 360
 ggccgggcgg cttgggggga acgggtggcn ggcatctgcc gctgcgaaga cngccttggg 420
 tccgccc 428

<210> 58
<211> 362
<212> DNA
<213> Homo sapiens

<220>
<221> misc_feature
<222> (1)...(362)
<223> n = A,T,C or G

<400> 58
 cgcccgccaa gaaggccgcg cccgcgaaga agggcgctcag ccgcgtcgtt ggcagcaaga 60
 caccggccac caagaccatc aaggncggcg cggccaagcc ggtggcgaag aaggcggctc 120
 cggccaagaa ggcgtgctccg gccanaaagg ccgcgcgcgc caagaaggtc gtcgccacga 180
 aagccccggc caagaaggct gcagccaaga agggctgatg cgtctccttc tagtegecgt 240
 ggcccgagcg cagccggcct gggccgacac ggcctatgaa gacttcgcca agcgccttcc 300
 gcccagagctg aggtcgagc tgaaggccgt caaggccgag acacgcggca gcaagacggc 360
 cg 362

<210> 59
<211> 691
<212> DNA
<213> Homo sapiens

<220>
<221> misc_feature
<222> (1)...(691)
<223> n = A,T,C or G

<400> 59
 cgcccgctta gtcgcagggc ccgccaccgc agggctcgcg agcccactgg gcccgatgga 60
 gccgcgcgct gccggggcg tgcgcnanct cncgcggcg ggggcccggg ggcgctaacg 120
 gtcgcaaaac anttcgcgc cctggggccg gaggcggctc aacaccntga ctgcnacct 180
 acgagacccg tttaacctan tgcggngtgt gctggcgga ncccgcgccg cttnaagcaa 240
 taaccgngcc gccacgcgtg ctgcccgcgc cctgaggag cgggcccctg cctcccgcg 300
 ccccgagtcc cactgcnot ccgnatgtca angngcccg ccccggtncg gcccacnca 360
 cgttgagacg cnaacaaaac ccnacggcc aggtncagc ttnccaagct ttatttattg 420
 gcaaatttgg gccggccnnc cgcacggcan ccttcgagnc anccgcnag tgtgcaccaa 480
 tcccgcgatg gngntttaat cgtgtttttt cttttctgga tgatataaat attgaccgna 540
 cacttcntgn ttgntccagg gntttntttt gggggcccca aaagccgcat ttggcctttg 600
 ggggaanagg ngaaggttcc tgcctnccg nccnanatta naaaaaatng ggantcccc 660
 gggccngcag gaattttnt tncaactta n 691

<210> 60
<211> 120
<212> DNA
<213> Homo sapiens

<220>
<221> misc_feature
<222> (1)...(120)

<223> n = A,T,C or G

<400> 60
 cggccgtgag gatgttggtg cccacgtgcg ctgtctcccg ncagtgcggc aggatgggtg 60
 tgatcaacgt gccaccatg cccaggaagc tgagcaggaa gcccanaagc tgcacgggcc 120

<210> 61
 <211> 229
 <212> DNA
 <213> Homo sapiens

<400> 61
 cggccgtcag ccatacgtaat gacatgtctg tgggttgccc tgtgccgcca ggctgggctg 60
 tcggaagcac ccagegacgt gtctgtgggt ccgcccctg cgcagaggcc gggccatcgg 120
 aaacacctgc agtaaccgga gtgccctcgc tgatagccct tgttccgggg cctcgtcctg 180
 ggctgtgcag agctccagcc cttagcccccag cccagctgc aggcggccg 229

<210> 62
 <211> 400
 <212> DNA
 <213> Homo sapiens

<220>
 <221> misc_feature
 <222> (1)...(400)
 <223> n = A,T,C or G

<400> 62
 tggactcacc gcggtggcng ctgacgccag cgtcacgggc tccgaggggc cagcccgccc 60
 gaggccaggt agccgctgac gggcacctgc ttggccagga gctgggaggt gggcgtgttg 120
 agcgccatga cgggctggcc caccacctgg atgggcgcca ggcccagcgt ggcgcgcgtg 180
 gcacccccag ggctgccatt gggcaggcct tggaggcccg ggatgggctg cagtgtcaca 240
 ttgcccaggc ccacaggctg cagggaagggc tgcacactca gggccttgtt gaccacgtcc 300
 tggggcggca ccagggcctg gtgggtcagc acggtgggcg ggccctgcag cccagcagg 360
 tcggtgctgc tgggaagagg gcttggggcc cgcagccac 400

<210> 63
 <211> 123
 <212> DNA
 <213> Homo sapiens

<400> 63
 ccgcggtggc ggccgccccg tctgggaggt ggggagtgcc tctgcccagc cgccacaccg 60
 tctgggaggt gaggagcgcc tctacctggc agccccatct ggggaactgag gaggcgctct 120
 gca 123

<210> 64
 <211> 110
 <212> DNA
 <213> Homo sapiens

<400> 64
 cggccgggca gaggcgccca cttcccagac ggggcggcca ggcagaggcg ctccccacct 60
 ccagatgaa gggcggtgg gcagaggtgc tccccacctc ccaggcgggg 110

<210> 65
 <211> 332
 <212> DNA
 <213> Homo sapiens

<400> 65
 cggccgagat gcactcagat ttatgttgtg aatttggtat gttcaggtaa tttgatgggtg 60
 tattcttatg caatgagatc tggatgtcat ttctggttct gctaattaga acatctgtga 120
 ccttgatcaa gcaagaactt tctctcttgt ggacctcaca tctacaatt gtatatgttc 180
 ctgcatgtcc ctcagacact tttcgttttt cttcagtcct ttttcttttt gtcctttaga 240
 ttggataatt tctgatcttc tgagaatttt tttattatct gcaacttgct gggtttttct 300
 tagaatttca gttttatttt tgtatttttt ta 332

<210> 66

<211> 204
 <212> DNA
 <213> Homo sapiens

<400> 66
 aggggtgcct ctgcgcccta aagaaaccgg gggagcccca caaccctcc cccaccagga 60
 cactaaaagg caagctttcg gtacagttag acatcaaagc ctcctaggcc ctgagtcaaa 120
 ggtatagccg tgtaatatcc cagtgccagc tctccggctg cggggagcct ggcgcaaagc 180
 ttccaagcct tccttgttca aaaa 204

<210> 67
 <211> 678
 <212> DNA
 <213> Homo sapiens

<220>
 <221> misc_feature
 <222> (1)...(678)
 <223> n = A,T,C or G

<400> 67
 tggactcccc gcggtggatg ccgcccggggc agccgaggcg aggactgcgg ggagctgacg 60
 ggtgagtagg gcanggacgg gcagatgcag cgtncgttca tgtccaggct gccaccggct 120
 gccagccac cctgggaccg ctcttgaga gacagcttgc gaccggagag gtggggccgg 180
 gcctgggacc cggaggagtc aagggggacc tcttgccat cggcctccag gggccggcca 240
 cctgcagttt tggggcccag ctggagggtca gcagggtgga ctcacaacc cctgagttca 300
 ggtacagggg gctgtggaga caggcccacc caggctgacc ttcccanag ccttgctgtc 360
 acggagagga gggggcgctt gaggaanggc cacaatgcn ngagaggggg caatggcctg 420
 ngacaagatg gagaacagcc acccgttccc cagtacagcc aggtcangac acggatccca 480
 ncaagccctt tggatgggga gactgaggta cagctgatga ctcaccctat gtgataccag 540
 ctgtgagagc cggagtggg atgcanacac ggaggtggcc agtggncacc tncnaagact 600
 caacatccan ggcgatgacg ccaaacagtc aaggcgtnag aaccccnan annaagagtg 660
 agtgnccatt acctaata 678

<210> 68
 <211> 113
 <212> DNA
 <213> Homo sapiens

<400> 68
 cggccgggcg gaggggctcc tcactttctca gacagggcat tcggtcagag atgctcctca 60
 caccacagac ggggcggtgg ggcagaggcg ctcccacat ccagacgat ggg 113

<210> 69
 <211> 179
 <212> DNA
 <213> Homo sapiens

<220>
 <221> misc_feature
 <222> (1)...(179)
 <223> n = A,T,C or G

<400> 69
 cggccgcgga ccccgacct cgacccaaac tgcattgcggc tgaggacccc caagccaggc 60
 agacgccaat ccagacccca cgnnnnnnnn nnaaganagg tttttttgcc cttttgacgt 120
 ttgggagttc cagtttctt taatagtggg acctctttg ttncaaaaan nggnaanat 179

<210> 70
 <211> 835
 <212> DNA
 <213> Homo sapiens

<220>
 <221> misc_feature
 <222> (1)...(835)
 <223> n = A,T,C or G

<400> 70

cgcccggtgc	caaaggtcct	gtgtgccag	aagaagtga	tggtttnggc	caggtcaggc	60
agaaggacct	ggttgtggca	gcgtgacaa	gagagcacc	cagatccatc	ccttacaaaa	120
tgatcgaggg	gcttcttcca	gagggcaccg	tctggttccc	tgaggggagt	gcagcagccc	180
tgacatagcc	ttcaggagcc	gtggcagagc	tgacaggggg	accccgagcag	tgggggccctg	240
acaaggacga	ggtgcaccac	catggggcctt	cccactgaac	tctcggcgcc	aggacgagcc	300
aagggacggg	ggcgggcgccc	ancccanact	caagctcagg	tcccttgggt	cccccgggg	360
gacaccttcg	acagcaggtt	cctggggcca	ccttctgccc	cacaccatga	ganaaaaacat	420
tgacggacga	atttctnctt	tgccccgcag	cccacgccc	ctntttccaa	ggtaggccct	480
nggcccctgg	cccattgaac	gaacgggcaa	gccnattaag	ggcnggnntt	tntgggaann	540
cctggggggg	ccaanccctt	ttttggnntt	ctttggggcc	tggaacacct	cnaacaatng	600
ggccccctn	gggggggccc	tttttnaaag	ggaacccttt	ttcggggggg	gggtttgggtc	660
ttnggggggg	gnccccctgg	ggggggnggg	gggaatcaac	ttggcaaaaa	cttcggggna	720
aacctnnggg	gcttttttng	ggccccgttt	ttaaaaacta	agtggggaat	ccccccnggg	780
cttgaggagga	attcnatatt	caagncttat	tgantacccg	gtcgancttg	gnnggg	835

<210> 71
 <211> 757
 <212> DNA
 <213> Homo sapiens

<220>
 <221> misc_feature
 <222> (1)...(757)
 <223> n = A,T,C or G

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<210> 76

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caaatagcag	ggcttttttt	ttttcctttt	atctcacaag	accactgtct	gctacctaaa	180
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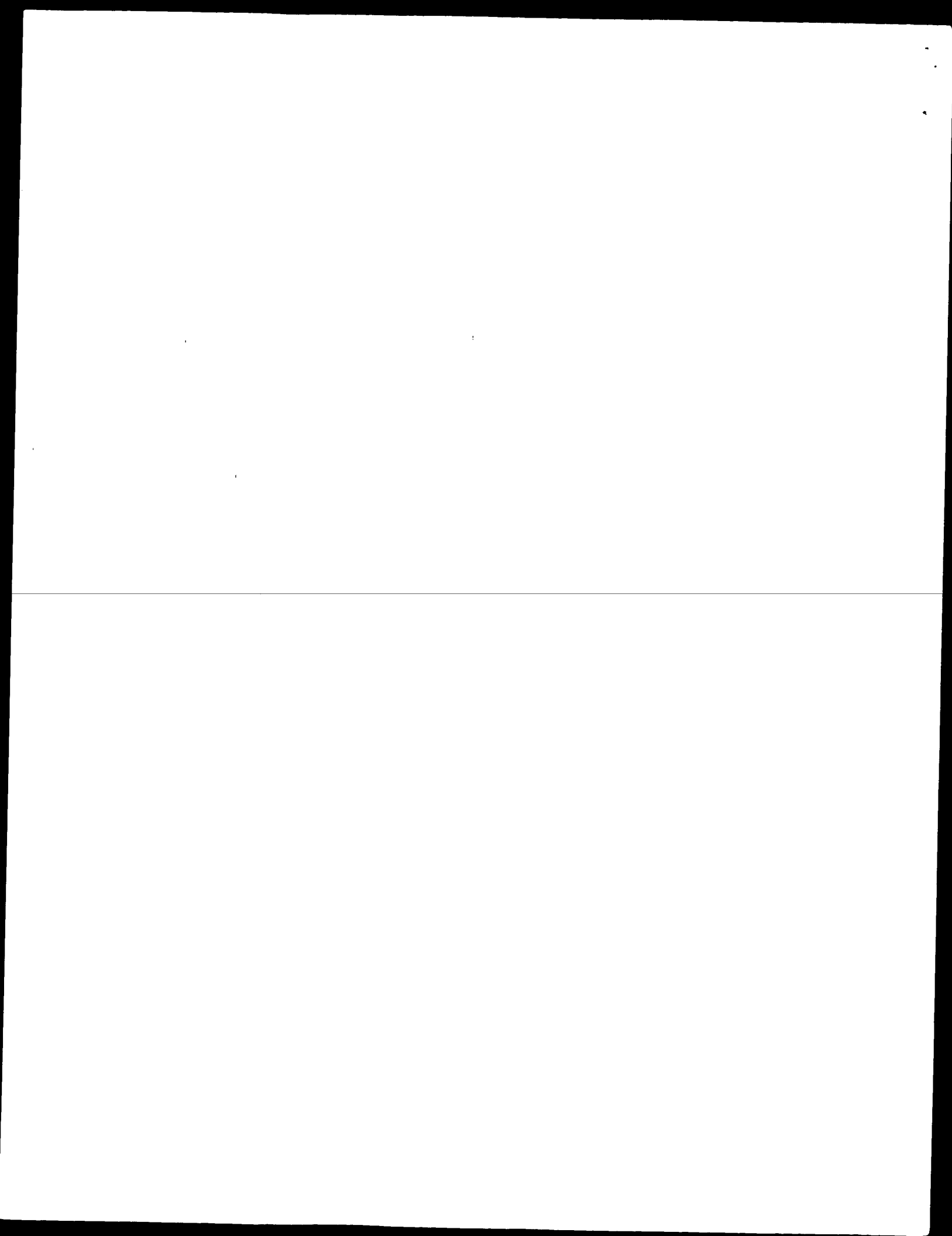
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GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC,
LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW,
MX, MZ, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK,
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patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE,
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(57) Abstract: Genomic imprinting is a parent of origin-dependent gene silencing that involves marking of alleles in the germline and differential expression in somatic cells of the offspring. Imprinted genes and abnormal imprinting have been implicated in development, human disease, and embryonic stem cell transplantation. We have established a model system for genomic imprinting using pluripotent 8.5 d.p.c. mouse embryonic germ (EG) cell lines derived from an interspecific cross. We find that allele-specific imprinted gene expression has been lost in these cells. However, partial restoration of allele-specific silencing can occur for some imprinted genes after *in vitro* differentiation of EG cells into somatic cell lineages, indicating the presence of a gametic memory that is separable from allele-specific gene silencing. We have also generated a library containing most methylated CpG islands. A subset of these clones was analyzed and revealed a subdivision of methylated CpG islands into 4 distinct subtypes: CpG islands belonging to high copy number repeat families; unique CpG islands methylated in all tissues; unique methylated CpG islands that are unmethylated in the paternal germline; and unique CpG islands methylated in tumors. This approach identifies a *methylome* of methylated CpG islands throughout the genome.

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INTERNATIONAL SEARCH REPORT

International Application No

PCT/US 01/16253

A. CLASSIFICATION OF SUBJECT MATTER

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A61P35/00

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B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 7 C12N C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal, WPI Data, PAJ, BIOSIS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

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X	WO 00 26401 A (UNIV JOHNS HOPKINS MED) 11 May 2000 (2000-05-11) page 6, line 1 -page 7, line 25 page 15, line 4-21 page 25, line 1 -page 28, line 8; example 8	46,60, 61, 63-73, 75-80
X	WO 00 04187 A (UNIV JOHNS HOPKINS) 27 January 2000 (2000-01-27) page 2, line 25 -page 4, line 21 page 14, line 24 -page 15, line 30 page 16, line 31 -page 17, line 28; example 1 --- -/--	46-50, 55-61, 63-80

☒ Further documents are listed in the continuation of box C.

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Date of the actual completion of the international search

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International Application No

PCT/US 01/16253

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
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A	MATSUI Y ET AL: "DERIVATION OF PLURIPOTENTIAL EMBRYONIC STEM CELLS FROM MURINE PRIMORDIAL GERM CELLS IN CULTURE" CELL, CELL PRESS, CAMBRIDGE, NA, US, vol. 70, 4 September 1992 (1992-09-04), pages 841-847, XP002912252 ISSN: 0092-8674 cited in the application page 841, left-hand column, paragraph 1 -right-hand column, paragraph 1 page 841, right-hand column, last paragraph -page 845, left-hand column, paragraph 2	1-3, 6, 8, 20-22, 25-27, 33, 40, 41
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INTERNATIONAL SEARCH REPORT

International Application No

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

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A	OVERALL MAREE ET AL: "Genomic imprinting in the rat: Linkage of Igf2 and H19 genes and opposite parental allele-specific expression during embryogenesis." GENOMICS, vol. 45, no. 2, 1997, pages 416-420, XP002191365 ISSN: 0888-7543 the whole document	46,55,58
A	SUTCLIFFE J S ET AL: "DELETIONS OF A DIFFERENTIALLY METHYLATED CPG ISLAND AT THE SNRPN GENE DEFINE A PUTATIVE IMPRINTING CONTROL REGION" NATURE GENETICS, NEW YORK, NY, US, vol. 8, September 1994 (1994-09), pages 52-58, XP002912861 ISSN: 1061-4036 abstract page 51	46,55, 58,68-73
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A	LEE C K ET AL: "Isolation and genetic transformation of primordial germ cell (PGC)-derived cells from cattle, goats, rabbits and rats." ASIAN-AUSTRALASIAN JOURNAL OF ANIMAL SCIENCES, vol. 13, no. 5, May 2000 (2000-05), pages 587-594, XP001061288 ISSN: 1011-2367 the whole document	1,6,8

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INTERNATIONAL SEARCH REPORT

International Application No

PCT/US 01/16253

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	<p>DATABASE BIOSIS 'Online! BIOSCIENCES INFORMATION SERVICE, PHILADELPHIA, PA, US; AN-PREV200000129707, GREGOROVA S ET AL: XP002191368 abstract & GREGOROVA S ET AL: "PWD/Ph and PWK/Ph inbred mouse strains of Mus m. musculus subspecies: A valuable resource of phenotypic variations and genomic polymorphisms." FOLIA BIOLOGICA (PRAGUE), vol. 46, no. 1, 2000, pages 31-42, abstract</p> <p>-----</p>	<p>1,2, 10-13</p>

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Information on patent family members

International Application No

PCT/US 01/16253

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		AU 1453700 A	22-05-2000
		EP 1127157 A1	29-08-2001
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